Biological Journal of the Linnean Society, 2009, **98**, 568–576. With 3 figures

Molecular characterization of an ancient *Hepatozoon* species parasitizing the 'living fossil' marsupial 'Monito del Monte' *Dromiciops gliroides* from Chile

SANTIAGO MERINO[1]*, RODRIGO A. VÁSQUEZ[2], JAVIER MARTÍNEZ[3], JUAN LUIS CELIS-DIEZ[2,4], LETICIA GUTIÉRREZ-JIMÉNEZ[2], SILVINA IPPI[2], INOCENCIA SÁNCHEZ-MONSALVEZ[3] and JOSUÉ MARTÍNEZ-DE LA PUENTE[1]

[1]*Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, J. Gutiérrez Abascal 2, E-28006 Madrid, Spain*
[2]*Instituto de Ecología y Biodiversidad, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile*
[3]*Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá, Alcalá de Henares, E-28871 Madrid, Spain*
[4]*Centro de Estudios Avanzados en Ecología y Biodiversidad, Departamento de Ecología, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile*

Received 12 March 2009; accepted for publication 8 May 2009

The Microbiotheriid *Dromiciops gliroides*, also known as 'Monito del Monte', is considered to be a threatened species and the only living representative of this group of South American marsupials. During the last few years, several blood samples from specimens of 'Monito del Monte' captured at Chiloé island in Chile have been investigated for blood parasites. Inspection of blood smears detected a *Hepatozoon* species infecting red blood cells. The sequences of DNA fragments corresponding to small subunit ribosomal RNA gene revealed two parasitic lineages belonging to *Hepatozoon* genus. These parasite lineages showed a basal position with respect to *Hepatozoon* species infecting rodents, reptiles, and amphibians but are phylogenetically distinct from *Hepatozoon* species infecting the order Carnivora. In addition, the *Hepatozoon* lineages infecting *D. gliroides* are also different from those infecting other micro-mammals living in sympatry, as well as from some that have been described to infect an Australian species of bandicoot. The potential vector of this parasite appears to be the host-specific tick *Ixodes neuquenensis* because the sequencing of a long amplicon determined the presence of one of the two lineages found in the marsupial. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **98**, 568–576.

ADDITIONAL KEYWORDS: hemoparasites – host-parasite coevolution – phylogeny – rodents – South America – tick.

INTRODUCTION

Among the American marsupials, there exists a species, the so-called 'Monito del Monte', *Dromiciops gliroides* Thomas, 1894, which is the last living representative of the Order Microbiotheria (Redford & Eisenberg, 1992; Herskhovitz, 1999). This small mammal is a tree climber species with nocturnal activity, which lives in southern forest of *Nothofagus* (Fagaceae) and *Chusquea* Bamboo in southern Chile and Argentina (Herskhovitz, 1999; Lobos *et al.*, 2005). The oldest fossil record from microbiotherids comes from the Early Paleocene of Bolivia (Gayet, Marshall & Sempere, 1991), and phylogenetic analyses of marsupials situated *Dromiciops* as being more closely related to Australidelphian than to Ameridelphians marsupials (Palma & Spotorno, 1999). It is clear that 'Monito del Monte' is a 'living fossil', being the last representative of a point of union between Australian and American marsupials.

*Corresponding author. E-mail: santiagom@mncn.csic.es

The protozoan blood parasites under the genera *Hepatozoon* are known to infect a variety of vertebrates, from anurans to mammals (Smith, 1996). These parasites are found in peripheral blood, where they infect white blood cells, except in marsupials and some rodents, where red blood cell infections are common (Smith, 1996). These parasites are transmitted to vertebrate hosts by ingestion of infected haematophagous ectoparasites, where the parasite is developed in the haemocoel. The variety of ectoparasites acting as definitive hosts of *Hepatozoon* parasites is also very diverse, varying from mosquitoes to mites, bugs, flies, lice, ticks, and fleas (Smith, 1996).

In the present study, we characterized, at the molecular and morphological level, *Hepatozoon* infections from a wild population of 'Monito del Monte' in Southern Chile. We also explored the phylogenetic position of these parasite lineages with respect to other previously described *Hepatozoon* species. In addition, we looked for the presence of these lineages infecting other micro-mammals cohabiting in the same area. Finally, we searched for potential parasite vectors using molecular tools.

MATERIAL AND METHODS

The study was conducted at Senda Darwin Biological Station and surrounding forest patches in northern Chiloé Island, Southern Chile. *Dromiciops gliroides* individuals were captured inside bird nest-boxes or by using Tomahawk-live traps (baited with banana), and blood samples were collected in August 2005 (for details of the study area, see Moreno *et al.*, 2005). Each animal was individually marked with numbered or coloured ear-tags to avoid resampling. Approximately 50 μL of blood were obtained with a heparinized hematocrit capillary tube from the tail vein or the infraorbital sinus (van Herck *et al.*, 2000). One part of the blood was immediately smeared on a slide, air dried, and later fixed with 96 ° ethanol and stained with Giemsa (1 : 10, v/v) for 40 min. Blood smears were scanned in search of blood parasites in accordance with previously described methods (Merino, Potti & Fargallo, 1997). The rest of the blood was transferred with the aid of a capillary to a sterilized tube containing a buffer composed of 100 mM Tris (pH 8.0), 100 mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulphate (SDS) (Jarvi, Schultz & Atkinson, 2002) to preserve blood samples for DNA analyses, or were stored on FTA classic cards (Whatman International Ltd.; Gutiérrez-Corchero *et al.*, 2002). To identify potential vectors of the *Hepatozoon* infections, animals were also captured three times a year in 2007 and 2008 (i.e. August, November, and February) and examined in search of ectoparasites. Ticks were the most common ectoparasite detected and collected (see Results). Ticks and two unidentified fleas were maintained alive for 24 h to allow the blood digestion and then conserved in 70% ethanol. Subsequently, ectoparasites were lyophilized to facilitate transport to the laboratory in Spain and DNA extraction. In addition, during the summer of 2007, in the same study area, we also captured and blood sampled the following rodents; two *Oligoryzomys longicaudatus* Bennett, 1832, 11 *Abrotrix (Chroeomys) olivaceus* Thomas, 1916, and four *Abrotrix sanborni* Thomas, 1916, to check whether the *Hepatozoon* species detected in *D. gliroides* were also infecting other micro-mammals in the area of study. Only blood samples for DNA analyses were obtained from these animals and were analysed as described below.

Parasite morphometric measurements were made with the aid of image analyser software (Scion Image) from pictures of parasites taken under a ×100 oil immersion objective using an Olympus BX41 optic microscope. The length and width of the parasite and parasite nucleus were measured for each parasite (Table 1). Infected and uninfected erythrocytes were also measured.

DNA ANALYSIS

Genomic DNA from *D. gliroides* samples conserved in lysis buffer was obtained using the UltraClean DNA BloodSpin kit (MO BIO Laboratories, Inc.). On the other hand, genomic DNA present in FTA cards was extracted to a soluble solution before polymerase chain reaction (PCR) using the following protocol: cored samples were transferred to collection vials

Table 1. Measurements of parasites and erythrocytes found in 35 *Dromiciops gliroides*

	Parasite	Parasite nucleus	Infected erythrocyte	Uninfected erythrocyte (ø)
Length (μm)	9.39 (0.64)	3.42 (0.43)	11.33 (2.07)	5.86 (0.50)
Width (μm)	2.59 (0.34)	1.95 (0.47)	4.57 (1.13)	–
Sample size	23	23	21	69

The diameter of uninfected erythrocytes is also presented. Standard deviations are shown in parenthesis.

Table 2. List of primers used in the present study

Primers	Sequences (5′- to 3′)
NBA1b	GTT GAT CCT GCC AGT AGT
NBA2	GCC TGC TGC CTT CCT TA
HEP1	CGC GCA AAT TAC CCA ATT
HEP4	TAA GGT GCT GAA GGA GTC GTT TAT
HPF1	CTA TGC CGA CTA GAG ATT G
HPF2	GAC TTC TCC TTC GTC TAA G
NBA1	GGT TGA TCC TGC CAG TAG T

with 250 μL of SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA; pH 8) at 4 °C for 6 h. Subsequently, SDS 20% (7 μL) and proteinase K (50 μg) were added to the vials and incubated at 55 °C overnight. After incubation, ammonium acetate 4 M (250 μL) was added to the vials at room temperature for 30 min. Subsequently, vials were centrifuged at 13 000 **g** for 10 min. After removing the pellet, DNA was precipitated with ethanol and resuspended in sterile water.

Amplification of 18S rDNA gene (1769 bp) was accomplished by PCR using the primers NBA1b/HPF2 (Table 2). PCR reactions consisted of 25-μL reaction volumes containing 20 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl, 1.5 $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems). The reactions were cycled at the following parameters using a thermal cycler (MasterCycler Personal, Eppendorf): 94 °C for 10 min (polymerase activation), 40 cycles at 95 °C for 40 s, 54 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplicons obtained after PCR assays were recovered from agarose gels and subjected to direct sequencing using the primers NBA1b, NBA2, HEP1, HEP4, HPF1, and HPF2 (Table 2). The sequencing was carry out using an ABI 3130 (Applied Biosystems) automated sequencer. DNA sequences were aligned and edited using CLUSTALW (Thompson, Higgins & Gibson, 1994) and BIOEDIT software (Hall, 1999), respectively. The phylogenetic analyses were performed using three different methods of inference after removing all columns containing gaps or missing data. The Neighbour-joining method (maximum composite likelihood model), the maximum likelihood method (GTR model; the gamma distribution parameter, the proportion of invariable sites, and the transition/transversion ratio were estimated) and Bayesian estimation (Markov chain Monte Carlo algorithm) were carried out using MEGA, version 4.0 (Tamura *et al.*, 2007), PHYML, version 3.0 (Dereeper *et al.*, 2008) and MrBayes (Ronquist & Huelsenbeck, 2003), respectively. Tree consistency was estimated by bootstrap analysis with 100 replications. All *Hepatozoon* sequences in GenBank with a similar size to those lineages found in *D. gliroides* were included in the phylogenies (Table 3). Although with some limitations, small subunit rDNA (SSU) is generally considered to be a good marker for addressing relationships of apicomplexan species and their history (Morrison & Ellis, 1997; Zhu, Keithly & Philippe, 2000; Šlapeta *et al.*, 2003). In addition, only SSU rDNA sequences are available for sufficient *Hepatozoon* species to make evolutionary inferences of parasites within this genus.

To detect the presence of the parasite in potential ectoparasite vectors, we followed the method of Schall & Smith (2006). This method aims to avoid the amplification of DNA from parasites digested with the blood meal. This allows the discrimination of whether a particular species acts as vector of a particular parasite because only parasites that survive to digestion are transmitted. Arthropods were maintained alive for 24 h, thus allowing for the blood meal to be digested, including the parasite DNA. Thus, it would be very difficult to amplify a long DNA fragment from the parasite, except if the arthropod is the vector. In this case, the parasites would be located in the haemocele, which is a safe place from digestive endonucleases.

The ticks and two fleas achieved from 'Monito del Monte' were maintained alive for 24 h before storage in 70% ethanol. DNA from lyophilized ectoparasites was extracted using the method previously described for FTA cards. Amplification of 18S rDNA gene (1096 bp) was accomplished by PCR using the primers NBA1/HEP4 (Table 2). PCR reactions were carried out using the conditions described above.

Statistical analysis

To identify potential differences in the prevalence of infection by *Hepatozoon* between individuals of different sexes and ages, we conducted statistical analyses using Yates corrected chi-square tests (STATISTICA, version 6.0; StatSoft, Inc.).

RESULTS

Overall, we captured 77 *D. gliroides*, comprising 44 males (39 adults and four juveniles; one male was not aged), 29 females (11 adults and 18 juveniles) and four individuals not sexed and aged. Excluding the last four individuals, blood smears were obtained from all animals. Also, with the exception of two juvenile females, blood for DNA analyses was obtained for a total of 75 individuals.

Thirty-five out of 73 (47.9%) blood smears from different individuals were found infected by a *Hepa-*

Table 3. Protozoan species used for phylogenetic analyses indicating their hosts, Genbank accession numbers, location, and reference when available

Parasite	Host	Genbank accession number	Location	Reference
Hepatozoon catesbianae	*Rana catesbiana*	AF 130361	USA	Carreno, Martin & Barta (1999)
Hepatozoon boigae	*Boiga fusca*	AF 297085	Australia	Unpublished
Hepatozoon sp.	*Isoodon obesulus*	EF152218 to EF152230	Australia	Wicks *et al.* (2006)
Hepatozoon sp.	*Abrotrix olivaceus*	FJ719815, FJ719817, FJ719818	Chile	Present study
Hepatozoon sp.	*Abrotrix sanborni*	FJ719816 FJ719819	Chile	Present study
Hepatozoon sp.	*Bandicota indica*	AB181504	Thailand	Unpublished
Hepatozoon sp.	*Clethrionomys glareolus*	AY 600625, AY 600626	Spain	Criado-Fornelio *et al.* (2006)
Hepatozoon felis	*Felis catus*	AY 620232, AY 628681	Spain	Criado-Fornelio *et al.* (2006)
Hepatozoon americanum curupira	*Dusycion thous*	AY 461377	Brazil	Criado-Fornelio *et al.* (2006)
Hepatozoon canis	*Canis familiaris*	AY 461378	Spain	Criado-Fornelio *et al.* (2006)
Hepatozoon canis	*Vulpes vulpes*	AY731062, AY 150067	Spain	Criado-Fornelio *et al.* (2006)
Hepatozoon canis curupira	*Dusycion thous*	AY 461375	Brazil	Criado-Fornelio *et al.* (2006)
Hepatozoon canis curupira	*Pseudalopex gymnocercus*	AY471615, AY 461376	Brazil	Criado-Fornelio *et al.* (2006)
Hepatozoon sp.	*Dromiciops gliroides*	FJ719813, FJ719814	Chile	Present study
Adelina bambarooniae	*Dermolepida albohirtum*	AF494059	Australia	Unpublished

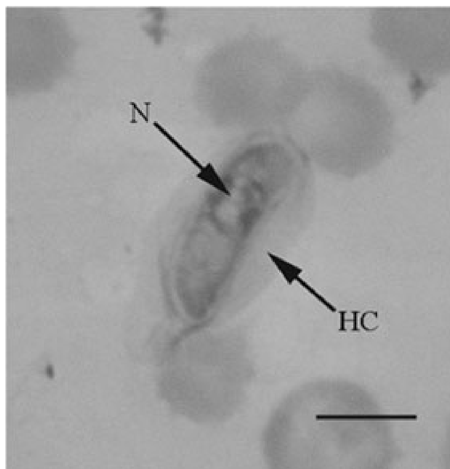

Figure 1. Photomicrograph of a blood smear from the 'Monito del Monte' (*Dromiciops gliroides*) showing an erythrocyte infected with *Hepatozoon*. N, parasite nucleus; HC, host cell. Scale bar = 5 μm.

tozoon species. Parasites infect red blood cells and show the typical banana shape of these parasite species (Fig. 1). Positive slides are deposited at the collection of Museo Nacional de Ciencias Naturales, Madrid, Spain (accession numbers: MNCN 35.02/33, MNCN 35.02/34, and MNCN 35.02/35 blood smears from *D. gliroides*). Molecular methods increased the prevalence of infection detected up to 86.7% (i.e. 65 out of 75 individuals infected).

There were no significant differences between sexes in the proportion of infected individuals estimated either by molecular ($\chi^2 = 1.52$, $P = 0.22$) or microscopic methods ($\chi^2 = 0.08$, $P = 0.78$). Significant differences between age classes were only found for data based on blood smears ($\chi^2 = 14.20$, $P < 0.001$), indicating that more adults than juveniles were uninfected. The fact that this difference was not significant for molecular data on infection ($\chi^2 = 0.01$, $P = 0.96$) indicates that the intensity of infection is higher in juveniles because many infections were not detected in adults by microscopic as compared to molecular methods. Differences in the prevalence of infection between sexes by age classes were not significant (data not shown, $P > 0.05$).

The tick *Ixodes neuquenensis* Ringuelet 1847 was collected from *D. gliroides* (two adults and 15 nymphs in 2007 and 13 adults and 12 nymphs in 2008). Long sequences from the *Hepatozoon* lineages infecting 'Monito del Monte' were recovered from three adult *I. neuquenensis* (one from 2007 and two from 2008). We

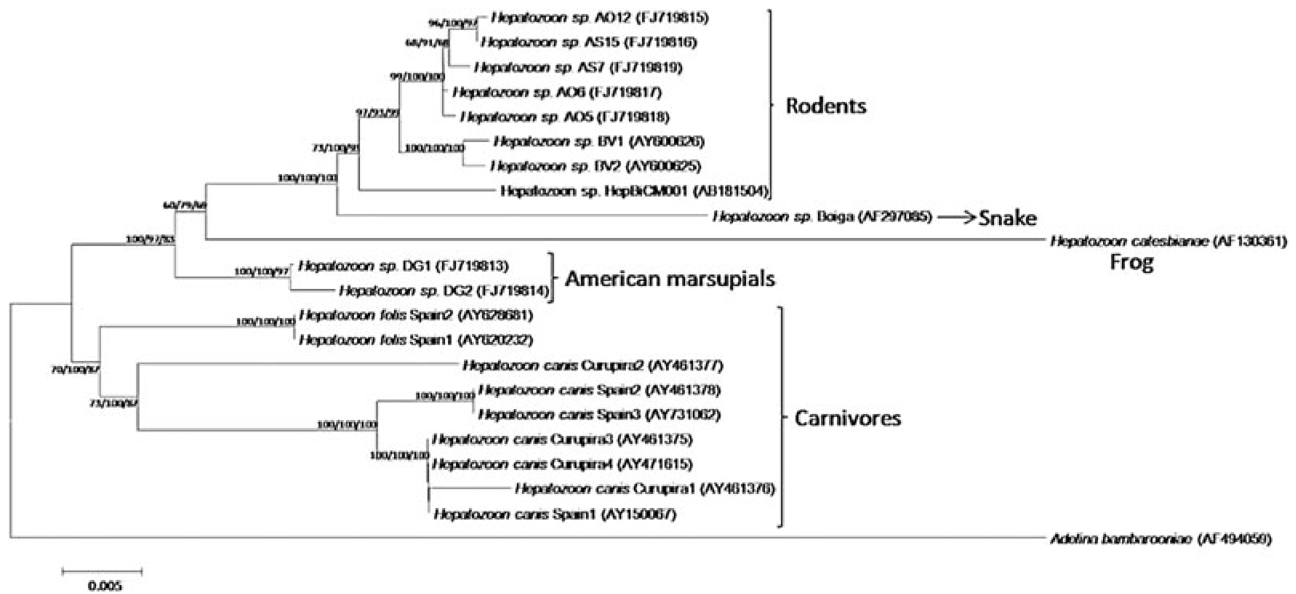

Figure 2. Phylogenetic tree obtained with the MEGA, version 4 software, using the Neighbour-joining method. Bootstrap values are shown at the corresponding nodes. When three values appear, they correspond to maximum composite likelihood, Bayesian, and maximum likelihood methods, respectively. Figures in parentheses after the species name indicate the GenBank accession number of the isolate. The hosts where *Hepatozoon* species were recovered are indicated. Samples from 'Monito del Monte' comprise those from American marsupials. This analysis is based on 1627 bp.

were unable to recover long segments of *Hepatozoon* from the two unidentified fleas.

Finally, we detected *Hepatozoon* infections in two of the three rodent species co-existing in the same area with 'Monito del Monte'. We found six *Abrotrix olivaceus* and two *A. sanborni* infected by five different *Hepatozoon* lineages. Three of them were present in *A. olivaceus* and two in *A. sanborni*. None of them were found infecting the 'Monito del Monte' (see sequences with accession numbers FJ719815 to FJ719819 in Fig. 2; Table 3).

Phylogenies

The tree including the short sequences of *Hepatozoon* species from the Australian bandicoot are based on 743 bp after removing columns containing gaps or missing data. This tree is used to determine the phylogenetic relationships between *Hepatozoon* lineages isolated from 'Monito del Monte' and those from the bandicoot (Fig. 3), and we infer phylogenetic relationships among parasites infecting different host taxa from the tree based on 1627 bp (see below; Fig. 2) although conclusions did not vary considerably by using either of them except for bootstrap support. The *Hepatozoon* lineages found in both marsupial species are not closely related between them and the lineages found in Australian marsupials, American marsupials/anurans, rodents/reptiles, and carnivore species form clearly separated groups (Fig. 3). To infer a sound phylogenetic relationship, we consider the more robust tree based on 1627 bp after removing columns containing gaps or missing data (Fig. 2). The topology of the tree indicates the grouping of the lineages in two clades supported with a high bootstrap value. One of them is formed by all *Hepatozoon* lineages found in carnivore species and the other by the lineages found in rodents, amphibians, reptiles, and the American marsupial. In this case, the lineages found in marsupials have a basal position in the clade.

DISCUSSION

The *Hepatozoon* species infecting 'Monito del Monte' appears to be clearly separated from lineages recovered from the Australian bandicoot. This may be interpreted as indicating that different *Hepatozoon* lineages developed independently in marsupials in both continents because the lineages recovered from *D. gliroides* appear to evolve from a common ancestor, previous to the separation of *Hepatozoon* species infecting rodents and *Isoodon obesulus*, although this tree topology lacks strong support (Fig. 3). Indeed, it has been recently suggested that parasite–host coevolution implying Apicomplexa follows the definitive hosts, namely those where sexual reproduction appear (Šlapeta *et al.*, 2003). Based on molecular and paleontological data, Palma & Spotorno (1999) pro-

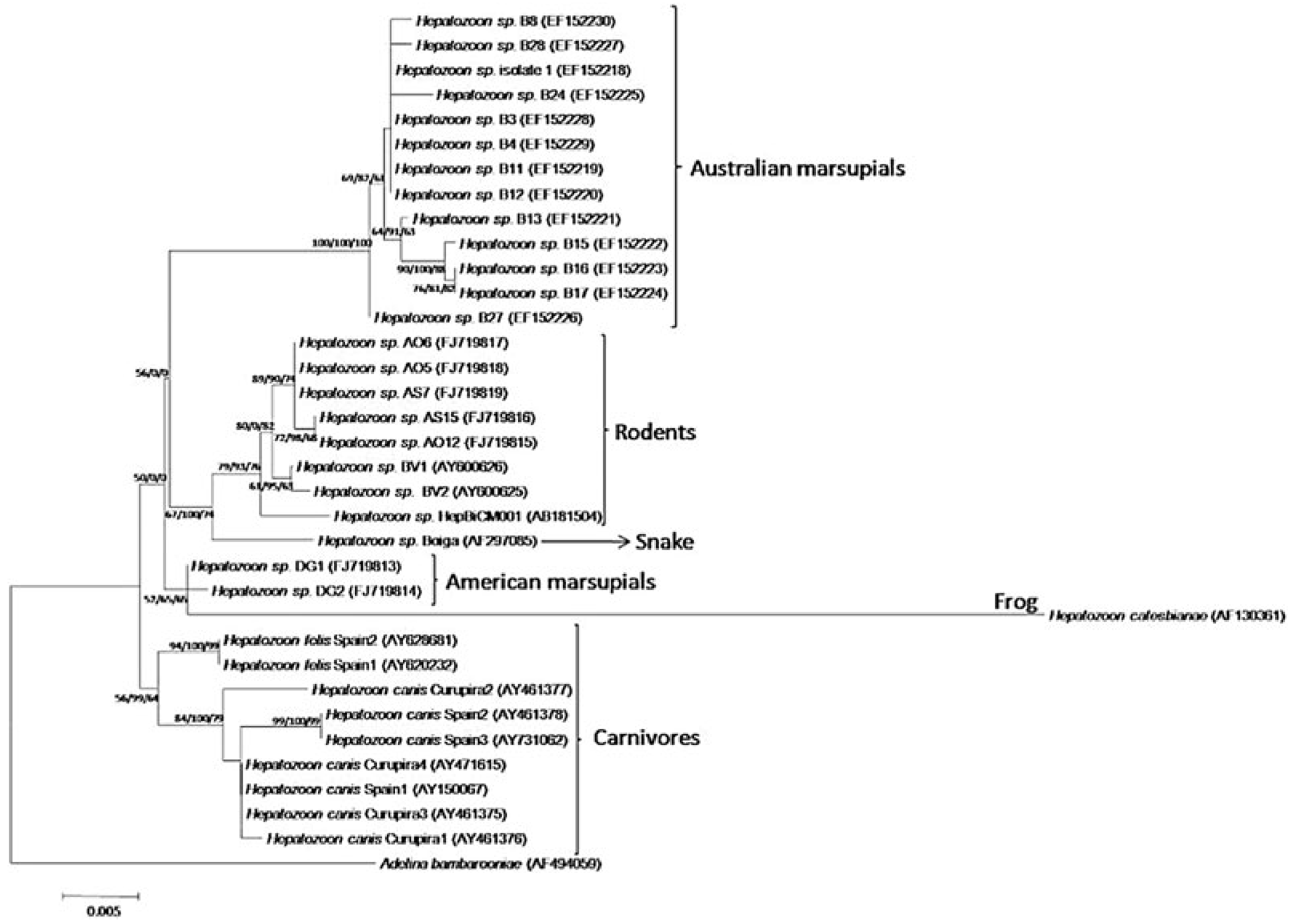

Figure 3. Phylogenetic tree obtained with the MEGA, version 4 software, using the Neighbour-joining method. Bootstrap values are shown at the corresponding nodes. When three values appear, they correspond to maximum composite likelihood, Bayesian, and maximum likelihood methods, respectively. Figures in parentheses after the species name indicate the GenBank Accession number of the isolate. The hosts where *Hepatozoon* species were recovered are indicated. Samples from 'Monito del Monte' are those from American marsupials. Samples from Australian marsupials are reported by Wicks *et al.* (2006). This analysis is based on 743 bp.

posed that Microbiotherids evolve in South America and later migrate to Australia across Antarctica prior to the separation of continents. If *Hepatozoon* species evolve with their definitive hosts and if *Hepatozoon* in *D. gliroides* separated from a common ancestor of other *Hepatozoon* species infecting Australian marsupials (Fig. 3), we can propose that the relationships *Hepatozoon–D. gliroides* is the result of a long coevolving relationship between host and parasite. That is, the relationship may have existed for a long time subsequent to the separation of continents. The complete sequence of 18S rDNA of *Hepatozoon* lineages from Australian marsupials may help to solve the position of the common ancestor of these parasites.

The possibility that *Hepatozoon* species infecting American marsupials were the ancestor to species infecting other mammals except carnivores obtains some reinforcement from the tree based on a longer fragment (1627 bp) of the small subunit ribosomal RNA gene, because the *Hepatozoon* lineages infecting *D. gliroides* appear in a basal position with respect to species infecting anurans, ophidians, and rodents, including lineages isolated from sympatric rodents. The *Hepatozoon* species parasitizing carnivores appear to be clearly separated in another clade (Fig. 2). Thus, the lineages isolated from *D. gliroides* appear to be host-specific and to be an ancient species of *Hepatozoon* with respect to those infecting rodents and lower vertebrates. The basal position of *Hepatozoon* in 'Monito del Monte' with respect to those in anurans and ophidians may indicate that these lower vertebrates were infected secondarily from mammals. *Hepatozoon* species present in rodents in the area also form a robust clade; thus, lineages of this parasite in these mammals also appear to be endemic to the area.

The basal position of *Hepatozoon* infecting 'Monito del Monte' prompted us to consider that the relationship between the marsupial and the parasite should also be mediated by an ancient and host-specific ectoparasite. In this respect, we recovered long segments of *Hepatozoon* DNA corresponding to the species infecting *D. gliroides* from three ticks using the method described above (Schall & Smith, 2006). Therefore, *I. neuquenensis* appears to be a potential vector of the *Hepatozoon* species infecting 'Monito del Monte'. This is interesting because *I. neuquenensis* is considered to be a host-specific ectoparasite of this vertebrate (Guglielmone *et al.*, 2004; Guglielmone & Nava, 2005; Marín-Vial *et al.*, 2007). However, this tick did not appear as a very ancient species compared to tick species from other vertebrates (Guglielmone *et al.*, 2006), although these data are only based on phylogenetic analyses of sequences of 460 bp. This may imply that the *Hepatozoon* parasite has adapted to *I. neuquenensis* later than to *D. gliroides*, and that either we failed to sample the original vector or it has now disappeared. The possibility that *I. neuquenensis* may have had a broad specificity and only recently invaded *D. gliroides* is not supported by the lack of reports of this tick species infecting other hosts (Guglielmone & Nava, 2005). It is possible that other vectors of the parasite exist because the prevalence of these *Hepatozoon* lineages in *I. neuquenensis* is very low compared to the prevalence in the vertebrate host. However, this may be related to host susceptibility and not necessarily to a relationship between high prevalences of infection in vertebrate hosts and a high abundance of vectors (Sol, Jovani & Torres, 2000).

Several species of *Hepatozoon* have been described infecting marsupials both in Australia and America, and all of them infect erythrocytes. A common species infecting American marsupials and described as *Hepatozoon didelphydis* was recently found to be a Sarcocystidae (Merino *et al.*, 2008), and a similar case has been recently reported for a marsupial species in Australia (Zhu *et al.*, 2009). Criado-Fornelio *et al.* (2006) failed to find *Hepatozoon* infections in 15 opossums (*Didelphis albiventris*) from Brazil either by molecular methods or microscopy, in contrast to the 25% prevalence detected by microscopy in the same species from French Guyana (de Thoisy *et al.*, 2000). *Hepatozoon* infections have also been reported infecting other neotropical marsupials, including *Didelphys marsupialis*, *Philander opossum*, and *Metachirus nudicaudatus* (d'Utra e Silva & Arantes, 1916; Regendanz & Kikuth, 1928; Garnham & Lewis, 1958; Deane & Deane, 1961; Ayala *et al.*, 1973). However, these reports are only based in microscopy and some of them are attributed to *H. didelphydis*, and may correspond to infections by an erythrocytic sarcocystidae (Merino *et al.*, 2008).

The high prevalence of these parasites in 'Monito del Monte' implies that it is a common parasite in the area of study. The infection is more difficult to detect in blood smears from adult individuals, thus indicating that juveniles suffer from more intense infections. This implies that juveniles are more susceptible to the parasite and adults probably control the infection better. This pattern agrees with an increase in immune responses as animals age, although, in most cases, the disease is not completely cleared, as demonstrated by the molecular data. Alternatively, highly-infected young individuals could die as a result of infection or associated causes and only individuals that are able to control infection reach adulthood. Information on the effects of infection on *D. gliroides* and on variation in the prevalence between populations, as well as on other potential vectors, will help to reveal the real impact of these parasites on this threatened 'living fossil' species of marsupial because *Hepatozoon* infections have been shown to be pathogenic with respect to their hosts (Ewing & Panciera, 2003).

ACKNOWLEDGEMENTS

This work is supported by the Fundación BBVA project BIOCON06/109. Research was supervised by the Ethics Committee of the Faculty of Sciences, Universidad de Chile, under a permit issued by the Servicio Agrícola y Ganadero, Chile. We thank J. Hetz, Rene Quispe, and P. Marín-Vial for field assistance. This study forms part of the research activities of the Institute of Ecology and Biodiversity grants ICM-P05-002, PFB-23-CONICYT-Chile, and FONDECYT 1060186 to R.A.V. J.M.-P. is supported by a grant from 'El Ventorrillo-CSIC' field station. J.L.C.-D. and S.I. acknowledge support from CONICYT-Chile graduate fellowships AT-24050068 and AT 24060066, respectively. This study is a contribution to the research program of Senda Darwin Biological Station, Chiloé, Chile. Jessica Herrero helped with the laboratory analyses. Constructive comments made by an anonymous referee and Dr María D. Forlano greatly improved the manuscript.

REFERENCES

Ayala SC, D'Alessandro A, Mackenzie R, Angel D. 1973. Hemoparasite infections in 830 wild animals from the eastern Llanos of Colombia. *Journal of Parasitolgy* **59:** 52–59.

Carreno RA, Martin DS, Barta JR. 1999. *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85:** 899–904.

Criado-Fornelio A, Ruas JL, Casado N, Farias NAR, Soares MP, Müller G, Brum JGW, Berne MEA, Buling-Saraña A, Barba-Carretero JC. 2006. New molecular data on mammalian *Hepatozoon* species (Apicomplexa: Adeleorina) from Brazil and Spain. *Journal of Parasitology* **92:** 93–99.

Deane LM, Deane MP. 1961. Sobre dois hemocitozoários encontrados em mamíferos silvestres da Regiaõ Amazónica. *Revista do Instituto de Medicina Tropical de São Paulo* **3:** 107–110.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard J-F, Guindon S, Lefort V, Lescot M, Claverie J-M, Gascuel O. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* **36:** web Server issue W465–W469.

Ewing SA, Panciera RJ. 2003. American canine hepatozoonosis. *Clinical Microbiology Reviews* **16:** 688–697.

Garnham PCC, Lewis DJ. 1958. Demonstration. Some parasites from British Honduras. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **52:** 295–296.

Gayet M, Marshall LG, Sempere T. 1991. The Mesozoic and Paleocene vertebrates of Bolivia and their stratigraphic context: a review. *Revista Técnica Yacimientos Petrolíferos Fiscales Bolivianos Santa Cruz, Bolivia* **12:** 393–433.

Guglielmone AA, Nava S. 2005. Las garrapatas de la familia Argasidae y de los géneros *Dermacentor*, *Haemaphysalis*, *Ixodes* y *Rhipicephalus* (Ixodidae) de la Argentina: Distribución y hospedadores. *Revista de Investigaciones Agropecuarias* **34:** 123–141.

Guglielmone AA, Venzal JM, Amico G, Mangold AJ, Keirans JE. 2004. Description of the larva and redescription of the nymph and female of *Ixodes neuquenensis* Ringuelet, 1847 (Acari: Ixodidae), a parasite of the endangered species *Dromiciops gliroides* Thomas, 1847 (Microbiotheria: Microbiotheriidae). *Systematic Parasitology* **57:** 211–219.

Guglielmone AA, Venzal JM, González-Acuña D, Nava S, Hinojosa A, Mangold AJ. 2006. The phylogenetic position of *Ixodes stilesi* Neumann, 1911 (Acari: Ixodidae): morphological and preliminary molecular evidences from 16S rDNA sequences. *Systematic Parasitology* **65:** 1–11.

Gutiérrez-Corchero F, Arruga MV, Sanz L, García C, Hernández MA, Campos F. 2002. Using FTA cards to store avian blood samples for genetic studies. Their application in sex determination. *Molecular Ecology Notes* **2:** 75–77.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

van Herck H, Baumans V, Boere HAG, Hesp APM, van Lith HA, Beynen AC. 2000. Orbital sinus blood sampling in rats: effects upon selected behavioural variables. *Laboratory Animals* **34:** 10–19.

Herskhovitz P. 1999. *Dromiciops gliroides* Thomas 1894, last of the Microbiotheria (Marsupialia) with a review of the family Microbiotheriidae. *Fieldiana Zoology* **93:** 1–60.

Jarvi SI, Schultz JJ, Atkinson CT. 2002. PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. *Journal of Parasitology* **88:** 153–158.

Lobos G, Charrier A, Carrasco G, Palma RE. 2005. Presence of *Dromiciops gliroides* (Microbiotheria: Microbiotheriidae) in the deciduous forests of central Chile. *Mammalian Biology* **70:** 376–380.

Marín-Vial P, González-Acuña D, Celis-Diez JL, Cattan PE, Guglielmone AA. 2007. Presence of *Ixodes neuquenensis* Ringuelet, 1947 (Acari: Ixodidae) on the endangered Neotropical marsupial Monito del monte (*Dromiciops gliroides* Thomas, 1894, Microbiotheria: Microbiotheriidae) at Chiloé Island, Chile. *European Journal of Wildlife Research* **53:** 73–75.

Merino S, Potti J, Fargallo JA. 1997. Blood parasites of some passerine birds from central Spain. *Journal of Wildlife Diseases* **33:** 638–641.

Merino S, Vásquez RA, Martínez J, Celis-Diez JL, Martínez-de la Puente J, Marín-Vial P, Sánchez-Monsalvez I, Peirce MA. 2008. A Sarcocystid misidentified as *Hepatozoon didelphydis*: molecular data from a parasitic infection in the blood of the southern mouse opossum (*Thylamis elegans*) from Chile. *Journal of Eukaryotic Microbiology* **55:** 536–540.

Moreno J, Merino S, Vásquez RA, Armesto JJ. 2005. Breeding biology of the thorn-tailed rayadito (Furnariidae) in south-temperate rainforests of Chile. *Condor* **107:** 69–77.

Morrison DA, Ellis JT. 1997. Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18S rDNAs of Apicomplexa. *Molecular Biology and Evolution* **14:** 428–441.

Palma RE, Spotorno AE. 1999. Molecular systematics of marsupials based on the rRNA 12S mitochondrial gene: the phylogeny of Didelphimorphia and of the living fossil microbiotheriid *Dromiciops gliroides* Thomas. *Molecular Phylogenetics and Evolution* **13:** 525–535.

Redford KH, Eisenberg JF. 1992. *Mammals of the neotropics: the southern cone: Chile, Argentina, Uruguay, Paraguay*, Vol. 2. Chicago, IL: University of Chicago Press.

Regendanz R, Kikuth W. 1928. Sur les Hémogrégarines de 'gamba', (*Haemogregarina didelphydis*), de la 'quica', (*Haemogregarina metachiri* n. sp.) et sur *l'Haemogregarina ratti*. *Comptes Rendus des Séances de la Société de Biologie et de Ses Filiales* **98:** 565–567.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Schall JJ, Smith TC. 2006. Detection of a malaria parasite (*Plasmodium mexicanum*) in ectoparasites (mites and ticks), and possible significance for transmission. *Journal of Parasitology* **92:** 413–415.

Šlapeta JR, Modrý D, Votýpka J, Jirků M, Lukeš J, Koudela B. 2003. Evolutionary relationships among cyst-forming coccidian *Sarcocystis* spp. (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle. *Molecular Phylogenetics and Evolution* **27:** 464–475.

Smith TG. 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). *Journal of Parasitology* **82:** 565–585.

Sol D, Jovani R, Torres J. 2000. Geographical variation in blood parasites in feral pigeons: the role of vectors. *Ecography* **23:** 307–314.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software, Version 4.0. *Molecular Biology and Evolution* **24:** 1596–1599.

de Thoisy B, Michel J-C, Vogel I, Vie J-C. 2000. A survey of hemoparasite infections in free-ranging mammals and reptiles in French Guiana. *Journal of Parasitology* **86:** 1035–1040.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

d'Utra e Silva O, Arantes JB. 1916. Sobre uma hemogregarina da gambá. *Hemogregarina didelphydis* n. sp. *Memorias do Instituto Oswaldo Cruz* **8:** 61–64.

Wicks RM, Spencer PB, Moolhuijzen P, Clark P. 2006. Morphological and molecular characteristics of a species of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) from the blood of *Isoodon obesulus* (Marsupialia: Peramelidae) in Western Australia. *Systematic Parasitology* **65:** 19–25.

Zhu BY, Hartigan A, Reppas G, Higgins DP, Canfield PJ, Šlapeta J. 2009. Looks can deceive: molecular identity of an intraerythrocytic apicomplexan parasite in Australian gliders. *Veterinary Parasitology* **159:** 105–111.

Zhu G, Keithly JS, Philippe H. 2000. What is the phylogenetic position of *Cryptosporidium*? *International Journal of Systematic and Evolutionary Microbiology* **50:** 1673–1681.