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A Sarcocystid Misidentified as *Hepatozoon didelphydis*: Molecular Data from a Parasitic Infection in the Blood of the Southern Mouse Opossum (*Thylamys elegans*) from Chile

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ABSTRACT. The blood of 21 adult South American mouse opossums (*Thylamys elegans*) captured from April through August of 2005 in central Chile was examined for parasites. Light microscopic analysis of blood smears initially suggested that a highly pleomorphic *Hepatozoon* species typical of American opossums was infecting erythrocytes. Unexpectedly, amplification by PCR and sequencing of a DNA fragment of the small subunit rDNA combined with phylogenetic analyses indicated that the parasite is not a member of the suborder Adeleorina, which includes the *Haemogregarina* and *Hepatozoon* species, but that it is a clearly distinct member of the suborder Eimeriorina, which includes the cyst-forming family Sarcocystidae. Therefore, a reclassification of this unusual intraerythrocytic apicomplexan will require additional life cycle, microscopic, and molecular analyses.

Key Words. Central Chile, coccidium, marsupial, parasite, phylogeny, Yaca.

"ONCE upon a time, the taxonomy of coccidian parasites was a relatively simple affair . . .." That is the beginning of the debate initiated by Levine and Baker (1987) about the taxonomy of coccidia. Later, Frenkel, Mehlhorn, and Heydorn (1987) acknowledged the debate and stressed the fact that the morphology of oocysts was not sufficient to identify a coccidian species, and that identification required the knowledge of all stages in the life cycle along with biochemical and metabolic characteristics. This debate has not yet ended, and new tools are now employed to clarify the still complex taxonomy of these parasites. The use of DNA analyses to correctly assign a parasite to a group, genus, or even species is currently used with success (see e.g. Merino et al. 2006; Schrenzel et al. 2005) and although by itself it is clearly insufficient for a complete descriptive approach, it is a highly valuable tool to disentangle taxonomic problems. For example, many genera and species of Apicomplexa have been tentatively assigned to a specific taxonomic group based on the host species they infect and the characteristics of only one or some of the life cycle stages. The difficulty in clarifying some parasite life cycles implies that many species are still being assigned to incorrect taxonomic groups. The use of molecular methods helps in such cases as the differentiation of isosporids of mammals and non-mammals (Carreno and Barta 1999), the resurrection of some genera (Modrý et al. 2001), or the phylogenetic assignment of some poorly known coccidia (Garner et al. 2006).

The southern mouse opossum *Thylamys elegans* is a nocturnal, small (30 g) insectivorous South American marsupial that inhabits semi-arid and Mediterranean areas of western South America from Peru to central Chile (Meserve 1981; Palma 1997), where it lives in shrub and arboreal habitats (Gallardo-Santis, Simonetti, and Vásquez 2005; Palma 1997). This mammal has been reported to be a host of *Trypanosoma cruzi* (Rozas et al. 2005), but to our knowledge no other blood parasite has been reported for this species. However, other species of American opossums have been found to be infected by several parasitic protists, including species of *Sarcocystis* (Rosenthal, Lindsay, and Dubey 2001), *Leishmania* (Santiago et al. 2007), and *Hepatozoon* (de Thoisy et al. 2000). *Hepatozoon didelphydis* is a pleomorphic species reported from several American marsupials, but since the original description of the species as *Hemogregarina didelphydis* (d'Utra e Silva and Arantes 1916), its life cycle has never been determined.

Using the 18S rDNA gene sequence, we report the phylogenetic placement of *H. didelphydis* into the family Sarcocystidae, and its rather high prevalence (93.8%) within erythrocytes of the South American mouse opossum *T. elegans*.

MATERIALS AND METHODS

Twenty-one mouse opossums were captured with Sherman traps baited with a mixture of oat, carrot, and tuna fish from April to August 2005 in Rinconada de Maipu (70°53′W, 33°28′S, 450 m asl), Region Metropolitana, about 30 km southwest of Santiago de Chile. Every animal was sexed and a blood sample was obtained by retro-orbital puncture of the venous plexus (van Herck et al. 2001). This method resulted in noticeably less stress to the animals than tail vein puncture or toe clipping. Immediately after extraction, a drop of blood was smeared on a slide, air-dried, and later fixed with absolute ethanol and stained with Giemsa solution (1/10 v/v) for 45 min. The rest of the blood was stored inside a plastic tube in a cool box and later frozen at −80 °C until analyzed. In seven cases, blood was obtained only for one analysis, resulting in a final sample size of 16 blood smears and 19 aliquots of blood for molecular analysis.

Smears were checked microscopically at 1,000X under oil immersion in search of blood parasites as described in Merino, Potti, and Fargallo (1997). Positive slides for the intraerythrocytic apicomplexan species were selected for the study. Slides are deposited at the collection of Museo Nacional de Ciencias Naturales, Madrid, Spain (Accession numbers: 35.02/30, 35.02/31, and 35.02/32, blood smears from *T. elegans*).

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Parasite morphometric measurements were made with the aid of the image analyzer software (Scion Image, Frederick, MD) from oil immersion pictures of parasites using an Olympus BX41 optic microscope. Lengths and widths of each parasite cell body and nucleus as well as the diameter of uninfected erythrocytes were measured (Table 1). Intensity of infection was measured as the number of parasites per 2,000 erythrocytes (Godfrey, Fedynich, and Pence 1987). When less than one parasite per 2,000 erythrocytes is found, we count 10,000 erythrocytes.

Genomic DNA was obtained from the infected blood of *T. elegans* using the UltraClean DNA BloodSpin kit (MO BIO Laboratories Inc., Solana Beach, CA). Partial amplification of the 18S rDNA gene from the erythrocytic parasite used PCR primers NBA1b (5′ GTT GAT CCT GCC AGT AGT 3′) and HPF2 (5′ GAC TTC TCC TTC GTC TAA G 3′) (Criado-Fornelio et al. 2006). Each 25-µl PCR reaction contained 20 ng 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, Foster City, CA). The reactions were amplified using a thermal cycler (MasterCycler Personal, Eppendorf, Hamburg, Germany) at 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.

DNA fragments recovered from agarose gels were directly sequenced using an ABI 3130 (Applied Biosystems) automated sequencer. Nucleotide sequence data are available in the GenBank® database under the Accession number EU443095. DNA sequences were aligned using the CLUSTALW program (Thompson, Higgins, and Gibson 1994). Sequences were edited using a BIOEDIT program (Hall 1999). We used MODELTEST (Posada and Crandall 1998) for the selection of the model of nucleotide substitution that best fits the data. The model selected using the Akaike Information Criterium was GTR+I+G ($-\ln L = 11556.0088$, $K = 10$, AIC = 23132.0176) that correspond to the General Time Reversible model by Rodríguez et al. (1990) with proportion of invariable sites I = 0.2847 and variable sites (G) gamma distribution shape parameter = 0.5571. These estimates were used as likelihood settings in PAUP* (Swofford 1998) to construct the neighbor-joining tree. Additionally, we also use this estimates to obtain a maximum-likelihood tree but using PhyML (Guindon and Gascuel 2003). Another third tree was obtained using the parsimony method (PAUP*). Tree consistency was estimated by bootstrap analysis with 1,000 replications in all cases except for maximum likelihood where only 100 replications were performed.

RESULTS

Overall, blood smears showed that 15 of 16 (93.8% prevalence) mouse opossums were infected with parasites (Table 1 and Fig. 1–3). The average intensity of infection was 7.9 (11.9 SD; range 0–45; $n = 16$) per 2,000 erythrocytes. No other blood parasites were detected in blood smears.

Based on the screening by PCR, the 19 mouse opossums analyzed were found infected with the parasite using molecular methods, including the single microscopically negative blood smear. Overall, the 21 blood samples were found infected by at least one method; blood smears or molecular methods. Unexpectedly, when a 1.674-kb fragment of the 18S rDNA from seven isolates was analyzed, all isolates showed 97% sequence identity with *Cystoisospora belli*. Alignment of these sequences showed that all

Table **1.** Measurements of parasites and erythrocytes found in 21 southern mouse opossums (*Thylamys elegans*).

	Hepatozoon didelphydis[a]	Parasite	Parasite nucleus	Infected erythrocyte	Uninfected erythrocyte
Length	8–10	8.07 (1.05)	3.25 (1.04)	8.61 (0.85)	6.30 (0.61)
Width	4–6	4.40 (0.87)	3.24 (0.82)	6.32 (0.66)	—
Sample size	—	30	30	24	87

Measurements are in µm.

Standard deviations are shown in parentheses. Uninfected erythrocytes were considered as perfectly round and only one diameter was measured.

[a]Data from d'Utra e Silva and Arantes (1916) in which sample size was not given.

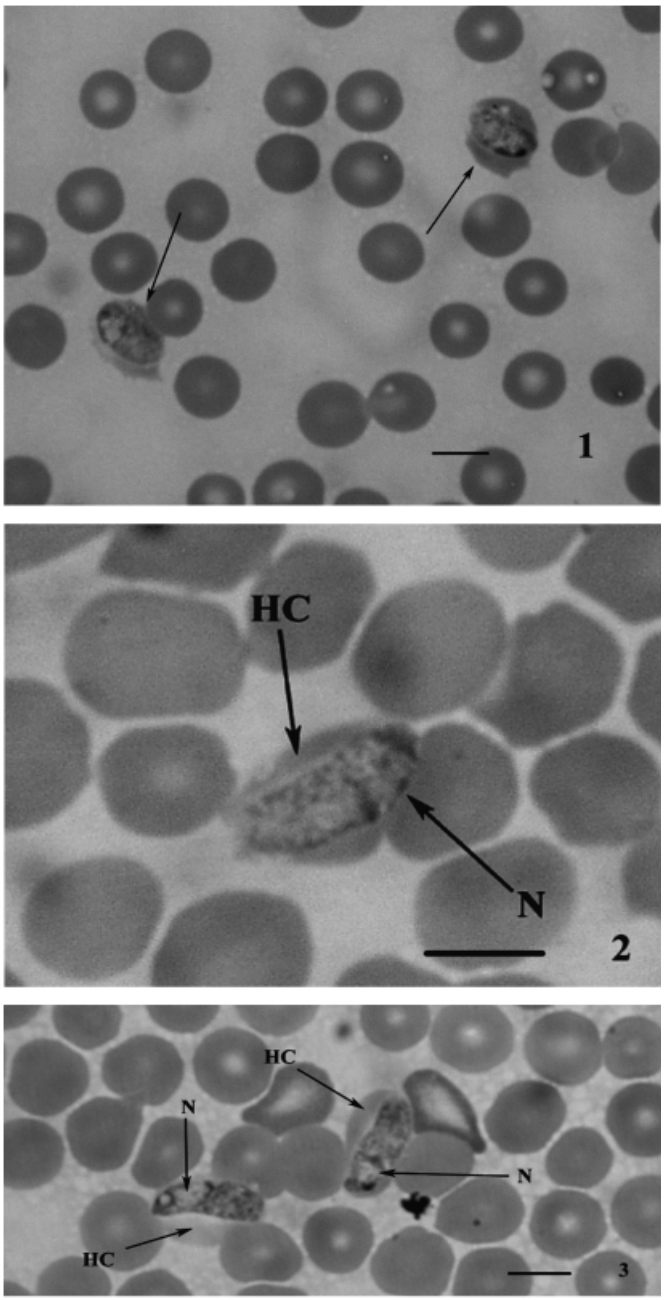

Fig. **1–3.** Photomicrographs of blood smears from the mouse opossum (*Thylamys elegans*) showing erythrocytes infected with coccidia. **1.** Red blood cells from the mouse opossum. Arrows indicate cells infected by the erythrocytic sarcocystidae. **2, 3.** Detail of parasites infecting blood cells. HC, Host cell; N, Parasite nucleus. Scale bar = 5 µm.

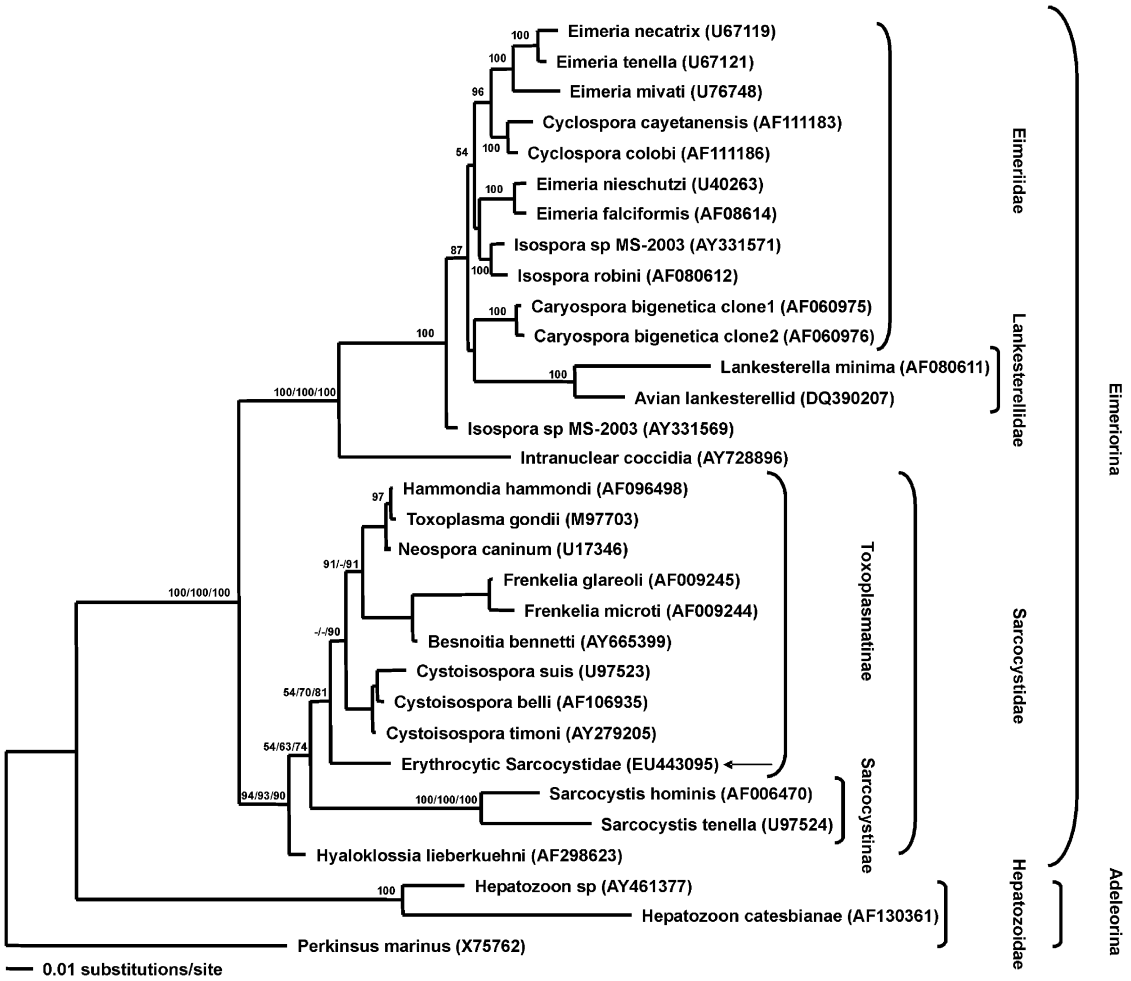

Fig. **4.** Phylogenetic tree obtained with the PAUP* software package using the neighbor-joining method. Bootstrap values are shown at the corresponding nodes. When three values appear, they correspond to maximum-likelihood, parsimony, and neighbor-joining methods, respectively. The absence of a value indicates lack of support for the node or bootstrap support below 50%. Figures in parentheses after the species name indicate the GenBank Accession number of the isolate. The coccidian species from the mouse opossum (*Thylamys elegans*) is named as erythrocytic Sarcocystidae (arrow).

seven isolates were identical. Phylogenetic analyses unequivocally related the parasites detected by PCR with the family Sarcocystidae (Fig. 4). The bootstrap values obtained were 94%, 93%, and 90%, respectively for maximum likelihood, maximum parsimony, and neighbor joining.

DISCUSSION

Hepatozoon didelphydis was first described from the big-eared opossum (*Didelphys aurita*) by d'Utra e Silva and Arantes (1916) as *Hemogregarina didelphydis* and later transferred to the genus *Hepatozoon* by Wenyon in 1926 (see Smith 1996). The parasite found infecting erythrocytes of the southern mouse opossum in this study is morphologically similar to that described by d'Utra e Silva and Arantes (1916) and presents the same characteristics of pleomorphism and color: fine alveolar pale blue cytoplasm containing a few red granules and a centrally placed nucleus lacking a clearly defined nuclear membrane (Fig. 1–3). Therefore, based solely upon morphology, we initially identified the parasite in *T. elegans* erythrocytes to be *H. didelphydis*. We can add to the initial description of this species the obvious fact that the form of the erythrocyte infected is altered in one direction as the width of infected and uninfected erythrocytes is similar, but their lengths are different (Table 1). The parasite shows a high prevalence of infection in the population under study as all the samples were found to be positive by at least one of the methods used (smears or PCR amplification).

Molecular characterization of the parasite, however, failed to confirm that this parasite is a *Hepatozoon* (or *Haemogregarina*) species as the phylogenetic study based on a fragment of the 18S rDNA grouped this parasite within the family Sarcocystidae. There exist several coccidia with extraintestinal stages within the Sarcocystidae and many of them include stages that go through host blood during the infection. However, to our knowledge, these stages are free in the blood or infect mononuclear cells; for example, *Sarcocystis cruzi* is found in the blood of intermediate hosts, but never within the erythrocytes (Dubey 1993, p. 84; Marquardt, Demaree, and Grieve 2000).

Several species of *Sarcocystis* infecting American opossum species are known (Rosenthal et al. 2001), but to our knowledge none from the southern mouse opossum (*T. elegans*). *Hepatozoon* species, probably *H. didelphydis*, have been reported infecting a number of neotropical marsupials, including *Didelphys marsupialis*, *Philander opossum*, and *Metachirus nudicaudatus* (Ayala et al. 1973; Deane and Deane 1961; de Thoisy et al. 2000; d'Utra e Silva and Arantes 1916; Garnham and Lewis 1958; Regendanz

and Kikuth 1928). However, identification of species of *Hepatozoon* is difficult based only on the morphology of blood stages. Molecular tools are required to help to uncover misidentifications among apicomplexan genera (see e.g. Merino et al. 2006). Therefore, it is not difficult to understand why this parasite might have been misidentified and assigned to *Hepatozoon.* In this respect, it is important to note that the high pleomorphism shown by the parasite infecting erythrocytes with round to elongate forms being found is uncommon within *Hepatozoon* infections; typically, variation is confined to slight changes in the length and width of the gamont, which always maintains a typical ''banana'' shape (Smith 1996).

The phylogenetic analyses place this parasite clearly among the family Sarcocystidae and grouped within the subfamily Toxoplasmatinae but with different support for the three different methods used. Using the neighbor-joining method, the erythrocytic sarcocystidae appear as an ancestral representative of both, the *Cystoisospora* species lacking the Stieda body (parasites of mammals; see Jenkins et al. 1999 for inclusion of these parasites in Toxoplasmatinae) and the ''heteroxenous'' coccidia (i.e. *Neospora*, *Hammondia*, *Toxoplasma*, and *Besnoitia*) (Fig. 4). However, using parsimony or maximum likelihood, the parasite appears as a sister taxon of these two groups. The entire group comprising the erythrocytic sarcocystidae, *Cystoisospora* species and ''heteroxenous'' coccidia have a high bootstrap support for the neighbor-joining and the parsimony method but not for the maximum likelihood (81, 70, and 54, respectively; see Fig. 4). On the other hand, Modrý et al. (2001) found strong support for monophyly of the subfamily Toxoplasmatinae and clades 1 (*Cystoisospora* species) and 2 (the ''heteroxenous'' coccidia), but reported instability for the position of *Hyaloklossia*. Here, we also found that instability as this taxon appears as an ancestral representative for Sarcocystidae using the neighbor-joining method but as a sister taxon to Toxoplasmatinae using parsimony and maximum likelihood. Additionally, the parasite infecting erythrocytes of *T. elegans* is clearly isolated from other Sarcocystidae, thus implying that it is a new genus within this family.

Traditionally, coccidia are split into two distinct groups. The first group (suborder Adeleorina) comprises coccidia of invertebrates and coccidia cycling between blood-sucking invertebrates and various vertebrates; this group includes *Hemogregarina* and *Hepatozoon*. The second group (suborder Eimeriorina) comprises coccidia of vertebrates as well as cyst-forming coccidia, including *Toxoplasma* and *Sarcocystis*. The fundamental difference between these two groups lies in their sexual development: syzygy for Adeleorina and independent gametes for Eimeriorina. We do not yet know the sexual development for the species studied here. Following phylogenetic analyses, the intraerythrocytic parasite found infecting *T. elegans* appears among the Sarcocystidae; so we expect that the life cycle of the parasite will be similar to parasites among this family: it should be a cyst-forming coccidian with a heteroxenous cycle not including vectors. However, there exists a coccidian parasite infecting erythrocytes of European moles (*Talpa europea* and *Talpa ceca*) that could be related to the erythrocytic parasite found in *T. elegans*. This parasite is *Elleipsisoma thomsoni*, which is transmitted by haemogamasid mites in which the isosporoid oocysts (typical for the Sarcocystidae) are formed (Mohamed, Molyneux, and Wallbanks 1987). Recently, the family Elleipsisomatidae was erected within Eimeriorina to accommodate this genus (Upton 2000), but unfortunately there are no molecular data available to enable us to relate this parasite to the one from *T. elegans*. Looking for parasite stages of the parasite from *T. elegans* in feces and in potential vectors (ectoparasites) may help to rule out some of these possibilities.

Thus, the parasite found infecting the blood of *T. elegans* appears to be misidentified as a *Hepatozoon* species (*H. didelphydis*) and clearly it should be assigned to the family Sarcocystidae. Samples from infections by *H. didelphydis* in other species should be analyzed in order to confirm that these infections have been assigned to the correct species. This is specially important as *Hepatozoon didelphydis* was described in other marsupial species and we cannot confirm completely that the parasite found in *T. elegans* is the same parasite. In addition, more information on the life cycle of the parasite is needed to confirm its generic status.

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