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VERSITA

Molecular characterization of arthropod-borne hematozoans in wild mammals from Brazil, Venezuela and Spain

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Abstract

A survey of *Babesia*, *Theileria* and *Hepatozoon* was conducted in wild mammals, including the capybara (*Hydrochaeris hydrochaeris*; n = 14) from Brazil, the jaguar (*Panthera onca*; n = 2) and crab-eating raccoon (*Procyon cancrivorus*; n = 4) from Venezuela, and the red deer (*Cervus elaphus*; n = 70), red squirrel (*Sciurus vulgaris*; n = 5) and Eurasian pine marten (*Martes martes*; n = 3) from Spain. Diagnostic procedures included both microscopy and molecular methods (PCR and sequencing of the 18S rRNA gene). Microscopic examination of blood smears revealed no hematozoan infections – unlike the molecular analyses. Nine Brazilian capybaras were found to be infected with *Hepatozoon canis* (prevalence 64%), two of which were coinfected with a previously unknown babesid (prevalence 14%) loosely related to *Theileria equi* (90% 18S rRNA gene similarity according to BLASTN® analysis).One jaguar and one crab-eating raccoon from Venezuela were infected by *H. canis*. Four of the red deer were infected with theilerids (5.7% prevalence), two with *Theileria* sp. and two with *T. annulata*. One red squirrel and three pine martens were infected with *Hepatozoon* sp. The isolate form the red squirrel was phylogenetically related to *Hepatozoon* sp. reported in Spanish bank voles, whereas those infecting the pine martens were related to *Hepatozoon felis* reported in Spanish cats. In conclusion, the molecular findings show that some non-canid mammals are carriers of *H. canis* in South America, while red deer may carry *T. annulata* in Europe. Small mammals in Europe appear to be unlikely hosts of *H. canis* and *H. felis*.

Keywords

Protozoa, *Babesia*, *Hepatozoon*, *Theileria*, phylogeny, wildlife

Introduction

Parasitic protozoa such as *Hepatozoon*, *Cytauxzoon*, *Babesia* and *Theileria* live in mammalian blood cells, sometimes causing severe disease and even the death of infected animals (Ewing and Panciera 2003, Jackson and Fisher 2006). According to recent molecular epizootiology studies, these hematozoans have wide geographic distributions (Baneth *et al*. 2000; Inokuma *et al*. 2002; Criado-Fornelio *et al*. 2003, 2006).

Wild animals act as a large reservoir of hematozoans that can, in some cases, infect man and domestic animals. For example, in Europe, humans have been infected with *Babesia divergens* (Centeno-Lima *et al*. 2003) perhaps from roe deer (García-Sanmartin *et al*. 2007), and in Brazil, dogs have been infected by *H. canis* carried by foxes (Paludo *et al*. 2005, Criado-Fornelio *et al*. 2006). Since wild mammals commonly show no clinical signs of disease (Mercer *et al*. 1988), molecular epizootiological studies of hematic protozoa in wild animals have been scarce. However, it is generally recognized that detailed knowledge of hematozoan vectors, carriers and reservoir hosts must be at our disposal if we are to develop and implement effective prevention and control strategies (Cortes *et al*. 2003).

This work reports a study of the molecular epizootiology of *Hepatozoon* and *Babesia/Theileria* in wild animals, performed by three associated laboratories examining blood and/or tissue samples from different geographic locations:

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southern Brazil (State of Rio Grande do Sul), northern Venezuela (States of Los Andes, Falcon, Los Llanos and Zulia), and central Spain (Castilla-León). These samples were collected from both native and introduced wild animals to gain insight into the pathogens currently affecting their populations, and to document potential reservoirs of disease that might affect man or domestic animals. To increase the sensitivity of hematozoan detection, two diagnostic methods were used: microscopy and PCR (targeting the 18S rRNA gene) followed by sequencing. The sequences obtained were subjected to phylogenetic analysis to determine the relationships among the isolates detected.

Materials and methods

Biological samples: The animals examined in this survey were selected on the basis of sample availability; all samples (blood and tissue from adult individuals) were kindly provided as part of different governmental animal health campaigns (see below). Venous blood samples (approx. 1 ml) were taken after the onset of anesthesia (see below) and collected in Vacutainer® (EDTA) tubes (Becton Dickinson, Franklin Lakes, NJ, USA). In South America, blood samples were freeze-dried before sending them to Spain by express courier service for molecular analysis. In Spain, blood samples from ruminants were centrifuged at 2000 *g* for 15 min at 4°C within 60 min of collection (i.e., in the field). The plasma was removed and the red blood cell pellets sent to the laboratory in a cold pack for analysis.

Fourteen capybaras (*Hydrochaeris hydrochaeris*, Caviidae) from southern Brazil (Pelotas, State of Rio Grande do Sul, approximately 31°42´S, 52°23´W) were examined; all were from a wildlife management area established by the Brazilian government. The animals were anesthetized (see below) to obtain blood and other biological samples, before being euthanized by authorized veterinarians.

In Venezuela (northern States of Los Andes – 8°50´N, 71°5´W; Falcon – 11°25´N, 69°41´W, Los Llanos – 5°56´N, 70°31´W and Zulia – 9°15´N, 72°44´W), blood samples were taken from two jaguars (*Panthera onca*, Felidae) and four crab-eating raccoons (*Procyon cancrivorus*, Procyonidae). Animals were anesthetized before sampling by authorized veterinarians.

In Spain, 70 blood samples were taken from wild red deer (*Cervus elaphus*) in the province of Soria (in the center-north of the country, approx. 41°43´N, 2°30´W). All were guided to a fenced area where they were immobilized before collection took place. They were released unharmed after sampling. This activity was conducted within the framework of an animal welfare program run by the local authorities. Finally, five red squirrels (*Sciurus vulgaris*, Sciuridae) killed in road accidents, and three pine martens (*Martes martes* – Mustelidae), victims of illegal hunting in the Province of Burgos (in the north-west of Spain, approx. 42°37´N, 2°40´W), were examined. Tissue samples were removed for microscope and molecular analysis as explained below. The time elapsed between death and sample processing was usually less than 24 h.

Anesthesia: The capybaras were anesthetized by electrical stunning (300V, 2A-5s). The crab-eating raccoons were captured using a conical net and then injected with an anesthetic. The jaguars were anesthetized using darts. The anesthetic consisted of a combination of xylazine 1 mg/kg and ketamine HCl (2 mg/kg for the jaguars, 1 mg/kg for the crab-eating raccoons). The same combination was employed with the red deer, although at a slightly different dose ratio (xylazine at 0.8 mg/kg, ketamine HCl at 2 mg/kg). Attempts were made to minimize disturbance to the animals before injections.

Microscopic diagnosis of hematic protozoa: Blood smears from the capybaras, jaguars, crab-eating raccoons and red deer were stained using standard Giemsa staining or the Hemacolor-Merck stain kit.

The internal organs of the squirrels and pine martens were removed and processed for histological examination. Whenever possible, lung, heart, striated and smooth muscle, liver and kidney samples were processed following standard histological procedures (hematoxylin-eosin staining). Stained slides or histological sections were examined at ×1000 using a Leica or Zeiss microscope.

DNA isolation and PCR assays: DNA was purified from mammalian blood using the Blood Spin kit (MOBIO, Solana Beach, California, USA). Liver or spleen tissue was used as a DNA source for the squirrels and martens (Criado-Fornelio *et al*. 2000)

Routine diagnosis of hematozoans was performed by PCR targeting the 18S rRNA gene. Fragments of approximately 400 bp were obtained for Piroplasmida (Criado-Fornelio *et al*. 2003) and 660 bp for *Hepatozoon* (Criado-Fornelio *et al*. 2006); these were then sequenced. The thermal cycling profiles, and the primers used have been reported elsewhere (Criado-Fornelio *et al*. 2003, 2006).

DNA analysis and sequencing: Products obtained by PCR were analyzed by initial electrophoresis in 5% polyacrylamide. Bands were visualized by ethidium bromide staining and UV transillumination. Band isolation was performed in 2% agarose gels using the Ultraclean 15 kit from Mobio. The DNA fragments obtained in at least two separate amplifications were sequenced using an ABI 3130 (Applied Biosystems Inc., Foster City, CA, USA) automated sequencer.

In positive samples genetically different from isolates previously described by ourselves or other authors, the complete 18S rRNA gene (approximately 1750 bp in *Hepatozoon* and 1700 bp in *Babesia*/*Theileria*) was sequenced. Sequencing was performed for at least three isolates whenever possible.

Phylogenetic analysis: DNA sequences were aligned using CLUSTALW® software (Thompson *et al*. 1994). Further manual alignment or determinations of pairwise percentages of sequence similarity were performed using the BIOEDIT® package (Hall 1999). For distance calculations and tree construction, the MEGA3.1® (Kumar *et al*. 2004), PHYLIP® (Felsenstein 1989) and TREECON® (Van de Peer and De Wachter 1993) software packages were used. Both phenetic (Jukes-Can-

tor, Kimura, Galtier and Gouy + neighbor joining) and cladistic (parsimony and maximum likelihood) procedures were followed in order to compare the topologies obtained. Tree-consistency was confirmed by bootstrap analysis (100 replications).

Results

Microscopic survey

No hematozoan infections were detected in any of the blood smears or histological sections.

Molecular survey

Hematozoans in Brazilian capybara: Table I shows the results of the molecular survey. Nine of the 14 capybaras (prevalence 64%) were infected with *H. canis* (genotype Pelotas 1 – GenBank® accession number EF622096; 99.9% identical to *H. canis* Curupira 1 – AY461376), two of which were coinfected with a hitherto undescribed protozoan (provisionally named "*Babesia* sp. capybara 1"). The sequence of the 18S rRNA gene of this new hematozoan, which was 90% identical to that of *Theileria equi* (Z15105) in BLAST® analyses, was deposited in the GenBank® database under accession number EF222255.

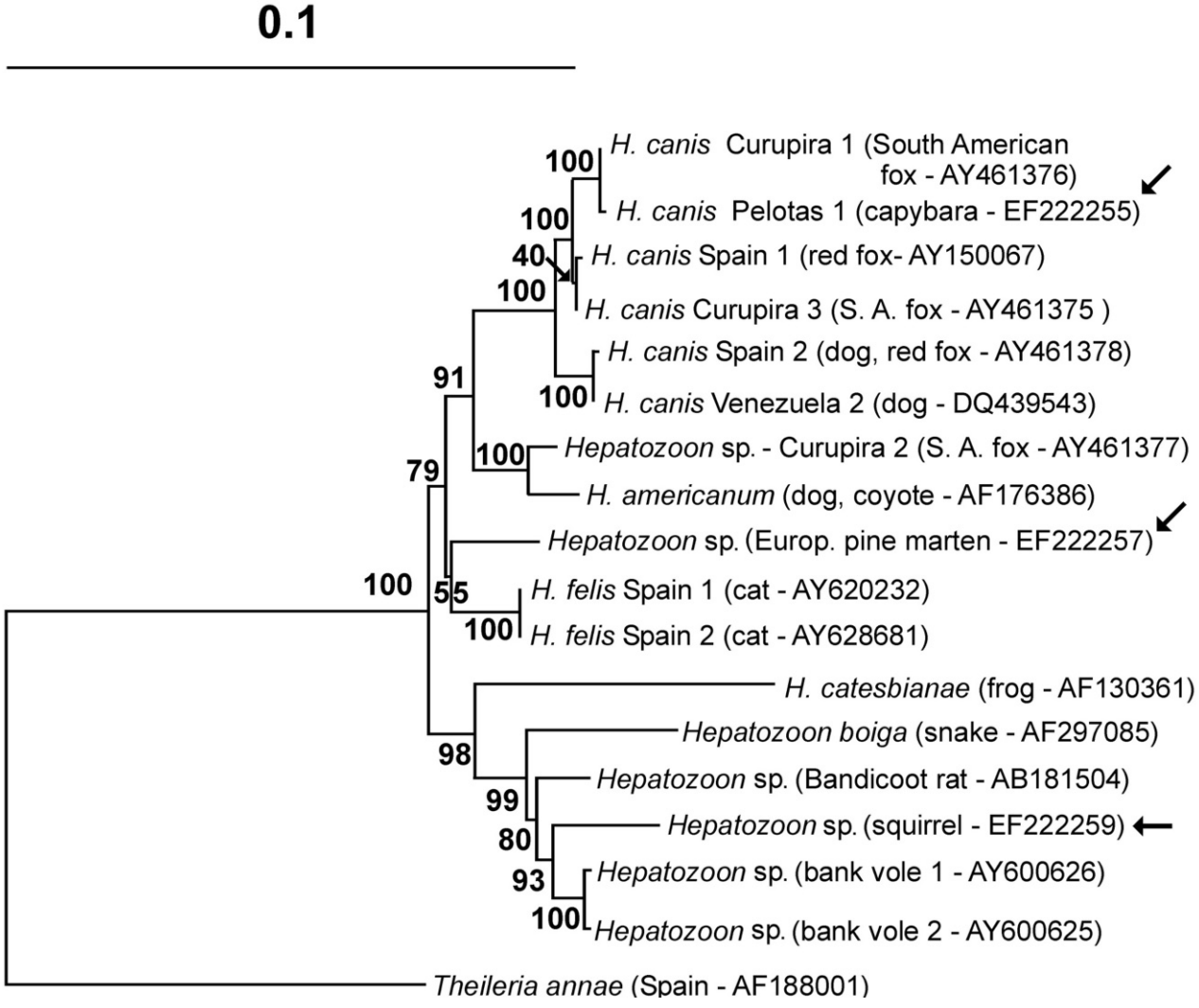

Fig. 1. Phylogenetic tree obtained for *Theileria annae* and 17 *Hepatozoon* species/isolates sequenced by the present and other authors. The method of Kimura (1980) was used to calculate distances; tree topology was inferred using the neighbor joining method. MEGA3.1® software was used for this analysis. Numbers at the nodes indicate bootstrap support obtained for 100 replications. The scale bar indicates an evolutionary distance of 0.1 nucleotides per position in the sequence. New sequences are marked by arrows. The GenBank® accession number and isolate name are indicated in brackets

Table I. Results of the molecular survey for hematozoans in wild mammals from Brazil (B), Venezuela (V) and Spain (S)

Mammals	Location	Infected	Hematozoans	Closest Genbank entry (by BLAST®) % sim.
Capybara	B	7	*H. canis*-Pelotas 1[a]-C[b] (1750 bp)	*H. canis* Curupira 1-AY461376-99%
Capybara	B	2	*Babesia* sp.-capybara 1[a]-C (1714 bp) + *H. canis*-Pelotas 1-P[b] (665 bp)	*Theileria equi*-Z15105-90% *H. canis* Curupira 1-AF461376-99%
Jaguar	V	1	*H. canis*-P (663 bp)	*H. canis* Kusadasy-DQ060324-100%
Crab-eating raccoon	V	1	*H. canis*-P (663 bp)	*H. canis* Kusadasy-DQ060324-100 %
Red deer	S	2	*T. annulata*[c]-P (404 bp)	*T. annulata* Spain-AF150056-100%
Red deer	S	1	*Theileria* sp.-P (401 bp)	*Theileria* sp. Spain-AY421708-100%
Red deer	S	1	*Theileria* sp.[c]-P (407 bp)	*Theileria* sp. Japan-AB012197-100%
Red squirrel	S	1	*Hepatozoon* sp. squirrel 1[a]-C (1764 bp)	*Hepatozoon* sp. bank vole 2-AY600625-97%
European pine marten	S	3	*Hepatozoon* sp. E. p. marten 1[a]-C (1757)	*Hepatozoon felis* Spain 2-AY628681-97%

[a]New sequence reported in the present paper. [b]C/P = complete or partial sequencing of the 18S rRNA gene. The sequence reading length is indicated in parentheses. [c]Species/isolates reported for the first time in red deer in Europe.

Hematozoans in Venezuelan mammals: The PCR results showed one jaguar and one crab-eating raccoon to be infected with *H. canis* (Table I) (prevalence is not indicated in this work when the study group contained <10 animals). Both isolates (GenBank® accession number EF222256) were identical and showed 100% similarity to *H. canis* Kusadasy (DQ060324).

Hematozoans in Spain: Four of the 70 red deer were infected with hematic protozoa (prevalence 5.7%, Table I): two with *T. annulata* (100% identical to AF150056), one with *Theileria* sp. (100% identical to AY421708 – isolate from *Cervus elaphus*), and one with *Theileria* sp. (100% identical to AB012197 – an isolate detected in *Cervus nippon*).

Finally, one red squirrel and three European pine martens were infected by a hematozoan. The sequence of the 18S rRNA gene of the red squirrel isolate (deposited in the GenBank® database under accession number EF222259) showed 97% similarity to *Hepatozoon* sp. detected in Spanish bank voles (AY600625). Finally, the three isolates from the European pine marten shared the same sequence (GenBank® accession number EF222257), which was 97% identical to that of *H. felis* detected in Spanish cats (AY628681).

Phylogenetic analysis of the 18S rRNA gene sequences: To study the phylogeny of the *Hepatozoon* spp., sequences of the 18S rRNA gene of 18 isolates were chosen (Fig. 1). *Theileria annae* was used as the outgroup. The alignment obtained with CLUSTAL® was 2398 bp long and contained 1171 phylogenetically informative positions. Phylogenetic trees constructed using MEGA 3 and TREECON® revealed that:

(i) *H. canis* Pelotas 1 from the capybaras and *H. canis* Curupira 1 (from South American foxes) form a robust group (Fig. 1). The separation among *H. canis* Spain 2/Venezuela 2 and other *H. canis* isolates (Curupira 3 and isolates from capybara and foxes from South America and Europe) enjoyed high bootstrap support.

(ii) The *Hepatozoon* sp. isolate from the Spanish squirrel was clearly related to the lineage of *Hepatozoon* species from rodents and lower vertebrates (Fig. 1).

(iii) The *Hepatozoon* sp. from the pine martens was related to those of the carnivore lineage (Fig. 1). However, due to the low bootstrap support for the node affecting this species, it is unclear whether it represents a sister lineage of *H. felis* or whether it is an unrelated branch. The *Hepatozoon* species from the pine martens appeared in the Galtier and Gouy-derived phylogram as basal to the entire carnivore lineage, but the node relating it to *H. felis* was not very robust (data not shown).

The sequences of 30 *Babesia*/*Theileria* species/isolates (one detected in the capybaras in the present work, and 29 from the GenBank database) were aligned for phylogenetic analysis. *Plasmodium falciparum* was used as the outgroup (Fig. 2). The alignment obtained was 2125 bp long and contained 688 informative positions. The phylogenetic affinities of the new babesid found in the Brazilian capybara could not be clearly determined. BLAST® analysis revealed a loose relationship between the piroplasmid capybara 1 and *T. equi*. However, in some of the phylogenetic trees constructed (see the Galtier and Gouy-NJ phylogram in Fig. 2) the Brazilian rodent isolate seems to be related to the babesids. The bootstrap support for this hypothesis was moderate (70) when the maximum likelihood method was used, and low (45) when the parsimony method was used (Fig. 2). The Kimura and Jukes Cantor phenetic procedures positioned *Babesia* sp. capybara 1 as a sister lineage of the babesids, but with very low bootstrap support (data not shown). Based on these data the new protozoan isolate from Brazil was provisionally identified as *Babesia* sp. capybara 1.

Discussion

In agreement with previous reports (Criado-Fornelio *et al*. 2007), the microscopic observation of blood smears failed to detect any hematic protozoa in what were later found to be PCR-positive animals. Microscopy would therefore appear to be an insufficiently sensitive method of diagnosing hematozoan infection.

The results show that South American capybaras, jaguars and crab-eating raccoons are potential carriers of *H. canis*. This agrees with the low host specificity shown by *H. canis* in South America, from where it has been reported in foxes (Cria-

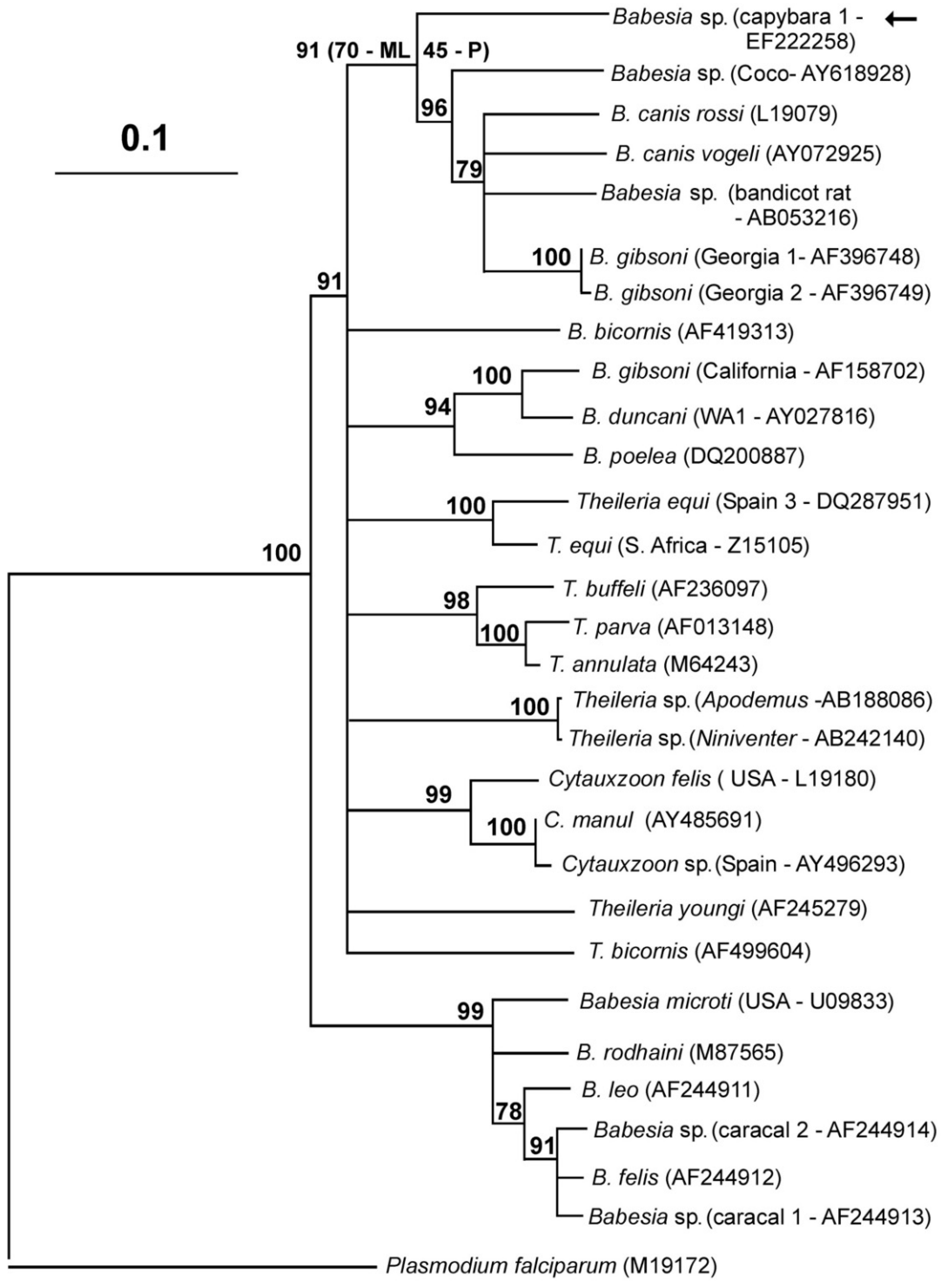

Fig. 2. Phylogenetic tree obtained for *Plasmodium falciparum* and 29 *Babesia*/*Theileria*/*Cytauxzoon* species/isolates sequenced by the present and other authors. TREECON® software was used for this analysis. The method of Galtier and Gouy (1995) was used for calculating distances; tree topology was inferred using the neighbor joining method. Numbers at the nodes indicate bootstrap support obtained for 100 replications. Numbers in parentheses at the node affecting *Babesia* sp. capybara 1 are the bootstrap values obtained with the maximum likelihood (ML) and parsimony (P) methods. Scale bar indicates an evolutionary distance of 0.1 nucleotides per position in the sequence. In this phylogram, nodes lacking biological significance (with bootstrap values of <70) were collapsed into polytomies. The new sequence is marked by an arrow. The GenBank® accession number and isolate name are indicated in brackets

do-Fornelio *et al*. 2006), dogs (Paludo *et al*. 2005) and cats (Rubini *et al*. 2006). This is the first time, however, that large felids have been shown suitable hosts.

Cervids have previously been reported carriers of theilerids in the Iberian Peninsula (Hofle *et al*. 2004, García-Sanmartín *et al*. 2007). However, the present study is the first to describe the occurrence of *T. annulata* in deer. It is evident that, in Spain (and probably elsewhere), cervids are carriers of some important cattle hematozoans. *Theileria* sp. (3185/02) from Spanish red deer has been reported from northern Spain (Hofle *et al*. 2004, García-Sanmartín *et al*. 2007), but a different *Theileria* sp. (CNY3A), identical to that found in *Cervus nippon* in Japan (Inokuma *et al*. 2004), was diagnosed by molecular methods in the present survey. Since *C. nippon* has never been introduced into Spain, the presence of *Theileria* sp. (CNY3A) in this country may explained by: (i) the geographic distribution of this protozoa including Europe, or (ii) the populations of *C. nippon* introduced into Central Europe inadvertently allowing transmission of this theilerid to European cervids. Whichever proves to be correct, the present data indicate the need for careful quarantine and health screening when translocating wildlife, as recently pointed out by Penzhorn (2006).

The *Hepatozoon* isolate found in the Spanish red squirrel is related to other *Hepatozoon* isolates from rodents (namely bank voles and the bandicoot rat), but clearly separated from the lineage that infects canids and felids. This conclusion is in agreement with the findings of Simpson *et al*. (2006) who partially sequenced the 18S rRNA gene of a *Hepatozoon* isolate from a red squirrel in England. Certainly, the decline in red squirrel populations in the UK has been associated with the presence of hematozoans (Simpson *et al*. 2006). In contrast, the species found in the European pine martens was closer to *Hepatozoon* isolates from carnivores. This probably reflects the existence of different definitive hosts (Carreño *et al*. 1997). In his review, Smith (1996) mentions no *Hepatozoon* species to infect the European pine marten. Therefore, the *Hepatozoon* isolate characterized by molecular methods in the present study probably represents a new species, although more morphological and biological data will be needed to support this hypothesis. The present study further confirms the suggestion that small mammals are unlikely carriers of canine/feline hematozoans in Europe (Criado-Fornelio *et al*. 2006).

This is the first time that babesids have been diagnosed by molecular methods in South American capybaras. Since some of the phylogenetic trees constructed related the Brazilian piroplasmida to babesids, this protozoan was provisionally identified as *Babesia* sp. capybara 1. Data obtained using the method of Galtier and Gouy were very important in this respect; this procedure is particularly useful for resolving problems arising through unequal base compositions (Galtier and Gouy 1995). The difficulties encountered when studying the taxonomic position of new *Babesia*, *Cytauxzoon* and *Theileria* isolates (Kjemtrup *et al*. 2001, Nijhof *et al*. 2003, Criado-Fornelio *et al*. 2004, Reichard *et al*. 2005) suggest that the phylogeny of these tick-transmitted protozoa is much more complex than previously thought. Further revisions in the status of piroplasmid taxa should be expected in the future (Allsopp and Allsopp 2006) as more genes and hematozoan isolates are studied in different hosts and geographic regions.

In conclusion, the present findings indicate that a number of wild animals are suitable carriers of hemoprotozoa in the countries studied. However, the importance of the new pathogens as potential agents of transmissible zoonotic diseases remains to be demonstrated. Further research will be needed if we are to fully understand the interactions between parasitic protozoa and host populations.

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