

# Characterization of *Hepatozoon* spp. in *Leptodactylus chaquensis* and *Leptodactylus podicipinus* from two regions of the Pantanal, state of Mato Grosso do Sul, Brazil

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**Abstract** *Hepatozoon* sp. are parasites that commonly infect frogs and arthropod vectors. This species has variability in the morphological and morphometric characteristics. Due to these variations, the naming of the species is thus impaired and only by visualizing the sporogonic cycle in vector and by molecular studies this problem can be solved. Recently, the use of molecular genetics has helped the species denomination. In this work, we collected 145 frogs (68 *Leptodactylus chaquensis* and 77 *Leptodactylus podicipinus*) in different sampling sites, where were found 18 (26.47 %) *L. chaquensis* and 24 (31.17 %) *L. podicipinus* parasitized; besides of gamonts, schizogonic forms were also seen in animals organs. The positivity difference between the collection sites for both frog species was not significant ( $p=0.958$ ). Comparing gamonts found in each species of anuran, we observed differences in morphology. The comparison in the molecular level for *L. podicipinus* was not possible due to small amount of blood obtained, just *L. chaquensis* had their parasites DNA sequenced. The amplified and sequenced samples, named HEP1 to HEP10, are presented in the phylogenetic tree as a different branch from other haemogregarines described on other hosts. Therefore, we have seen that, although the morphology and morphometry of the collected parasites at each site showed differences, the sequencing of these samples

revealed identical species of *Hepatozoon*, and different compared to those from GenBank, thereby demonstrating that the species of *Hepatozoon* in *L. chaquensis* observed in this study probably represent a new species.

**Keywords** Leptodactylidae · *Hepatozoon* sp · Frogs · *Leptodactylus chaquensis* · *Leptodactylus podicipinus*

## Introduction

The anuran amphibians have a complex life cycle that can be affected by many factors throughout its existence and can host ectoparasites and endoparasites. Among hemoparasites found, we can highlight the group of the haemogregarines, among them species of the *Hepatozoon* genus (Barta and Desser 1984).

This genus shows so diverse morphology in the blood of frogs that creates confusion among researchers when naming their found species, and different names were given to the same protozoan (Levine 1988; Desser et al. 1995; Smith 1996; Desser 2001). Costa et al. (1973) observed schizogonic forms in organs of *Leptodactylus latrans* and *Leptodactylus pentadactylus* and sporogonic forms in invertebrate (*Haementeria lutzi*). Desser et al. (1995) were the first to investigate the complete cycle of parasite development in vertebrates (*Lithobates catesbeianus*) and the sporogonic cycle in the vector (*Culex territans*). Posteriorly to this study, researchers such as Kim et al. (1998) and Harkness et al. (2010) used the experimental infections and reported morphological, morphometric, and molecular comparisons.

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Regarding to the problem of identifying the haemogregarines found in anurans, several researchers have proposed the molecular analysis of 18S ribosomal RNA (rRNA) sequences (conserved regions) (Haag et al. 1998; Martin et al. 2002) and ITS (variable regions) to infer phylogenetic relationships among these protozoan (Schlegel 1991; Cai et al. 1992; Maia da Silva et al. 2004; Rodrigues et al. 2006; Ferreira et al. 2007).

Our goal was to determine the occurrence of *Hepatozoon* sp. in frogs *Leptodactylus chaquensis* and *Leptodactylus podicipinus*, from the region of the Pantanal (Brazil), and characterize the parasites by morphology, morphometry, and molecular analysis, in order to infer the phylogenetic relationships among the samples, as well as among the different species already described in the available databases.

## Material and methods

The capture of specimens was performed under the approval of the Ethics Committee on Animal Experiments (CEEAA) no. 1608, located at the Biosciences Institute, São Paulo State University (UNESP), and authorized by the Brazilian Institute of Environment and Renewable Natural Resources IBAMA, no. 16696-1.

We collected 145 adults individuals, 68 *L. chaquensis* and 77 *L. podicipinus*. The animals were captured in the Pantanal Study Base (BEP), located in Passo do Lontra, municipality of Corumbá – Mato Grosso Sul State, Brazil (19° 34' 39" S, 57° 0' 44" W) and in the EMBRAPA Research Center (18° 59' 00" S, 56° 39' 00" W), in Nhumirim farm, located in the subregion of Nhecolândia, also in the municipality of Corumbá. These collection sites are located at a distance of approximately 76 km apart one of other. The subregion of Passo do Lontra included in the Paraguay River basin, located at the lower portion of the Miranda River subbasin, under influence of the Abobral River, with rainy season from November to March (Polizer et al. 2000).

Nhecolândia is characterized by a mosaic of saline and freshwater lagoons, interspersing cordilleras with forest vegetation, connected by natural channels that receive water only during major floods, delimiting lanes of Brazilian savannah and grassland vegetation (Rodela 2006). The region has two distinct seasons, the rainy, from November to March, and the dry, from April to October (Soriano and Alves 2005).

*L. chaquensis* is a very abundant species in open areas in the Pantanal and surrounding plateau, both males and females measure 7.1 cm, whereas *L. podicipinus*, also abundant in Pantanal, males measure only 3.5 cm and females 3.9 cm. (Uetanabaro et al. 2008).

The capture was performed manually at night, in April, November, and December 2008, and January 2009, and all animals were packed in plastic bags and transferred to the

laboratory. Subsequently, the animals were euthanized with a sodium thiopental injection; blood was collected from the heart to make the blood smears and the molecular analysis. The animals were necropsied and organs like the liver, spleen, and kidney were used to make the organs imprint, in order to visualize schizogonic stages of *Hepatozoon* sp.

All blood samples were frozen for molecular characterization by PCR. When it was not possible to collect blood, as in the case of very small specimens of *L. podicipinus*, the slides were scraped (Scopel et al. 2004; Sykes et al. 2008) and washed with lysis buffer (Qiagen) and the DNA extraction was performed normally. Blood smears and organs imprinting were prepared, fixed in methanol, and stained with Giemsa (10 %) for 30 min. The slides were examined under an optical microscope ( $\times 1000$  magnification) for the diagnosis of the parasite.

The morphology and the morphometry of the parasite were obtained by using the image analyzer software Qwin lite 2.5 (Leica). The observed morphometric variables of the parasite were the area (BA), length (BL), and width (BW) of its body, as well the area (NA), length (NL), and width (NW) of its nucleus.

The extraction of total DNA was performed by using the commercial kit QIAmp DNA Blood Kit (Qiagen) according to the manufacturer's instructions. The amplification of fragments of DNA samples was performed by means of HEMOI oligonucleotides 5'-TAT TGG GAATAA TTT TTA CTA ATT TGATTG-3' and HEMOII 3'-CCT TTA CTT CTT CTC AGT AAG GTT GAT CAC-5' (Perkins and Keller 2001) targeting the 18S rRNA region. The reactions were prepared for a final volume of 20  $\mu$ l containing 10  $\mu$ l of GoTaq Colorless Master Mix (GoTaq<sup>®</sup> DNA polymerase, GoTaq Colorless 2 $\times$  Reaction buffer, pH 8.5, 400  $\mu$ M dATP, 400  $\mu$ M dGTP; 400  $\mu$ M dCTP, 400  $\mu$ M dTTP, and 3 mM MgCl<sub>2</sub>) (Promega), 10 pmol of each primer (forward and reverse), 1  $\mu$ l of total DNA and H<sub>2</sub>O. All reactions were performed using a negative control.

Reactions were carried out using My Cycler (BioRad) thermocycler at the following conditions: initial cycle at 94 °C (hot start) for 3 min followed by 40 cycles of 94 °C for 45 s, 56 °C for 1 min and 72 °C for 1 min, with a final extension cycle at 72 °C for 7 min. The generated products were visualized on 1 % agarose gel stained with GelRed 0.5  $\mu$ g/ml (Biotium). The amplified fragments were compared with the 1 kb DNA Ladder (Fermentas) used as standard and viewed in UVDI transluminator (Major Science). Positive samples showed approximately 900 bp in size, which were subjected to sequencing. Among the positive samples, ten were sequenced and compared to the sequences of the GenBank using the BLASTn software (<http://www.ncbi.nlm.nih.gov/BLAST>). The complete alignment of sequences was performed using the ClustalX (1.83) software. Methods of distance (neighbor-joining (NJ)) were used to construct the phylogenetic tree (Saitou and Nei 1987), and the bootstrap

test was used to estimate the analysis consistency index of the branches distances of the NJ trees (Felsenstein 1985).

For the statistical analysis, we used the EPI-INFO software to perform the chi-squared test. The level of significance was set at  $p < 0.05$ . In this test, we verified whether there was a significant difference between the positivity of the species of frogs and their capture sites and also if there were significant differences of positivity between the species of frogs. For determination of variation of standard deviation in the morphology parameters of the parasitic species found in the frogs, we used the following formula (Costa Neto 1994):  $cv = 100\sigma/X$ , in which  $cv$  is the coefficient of variation,  $\sigma$  is the standard deviation, and  $X$  is the mean.

## Results

### Collection sites

In the first collection site (BEP), 73 frogs were captured (38 *L. chaquensis* and 35 *L. podicipinus*), while at Nhumirim farm, 72 frogs were captured (30 *L. chaquensis* and 42 *L. podicipinus*), totaling 145 animals captured, 68 *L. chaquensis* and 77 *L. podicipinus*.

### Positivity for *Hepatozoon* spp.

The positivity of the animals in relation to *Leptodactylus* species and collection site can be seen in Table 1.

Of the 68 captured *L. chaquensis*, 8 (11.76 %) were positive for smear microscopy of blood or organs, 4 from BEP and 4 from Nhumirim, while for PCR technique, 18 animals (26.47 %) were positive, 13 from BEP and 5 from Nhumirim. Among the *Hepatozoon* sp. samples positive for blood smears technique, three were false-negative, i.e., were negative by PCR even having gamonts and schizonts typically seen in *Hepatozoon* sp.

Among the 77 *L. podicipinus* captured, 2 (2.60 %) were positive for smear microscopy of blood or organs, both collected at Nhumirim, while for PCR, 24 (31.17 %) were

positive, 8 from BEP and 16 from Nhumirim. Among the positive samples on slides, only one was false-negative.

### Collection sites and positive animals for PCR

In BEP 73, animals were collected, of which 21 (28.77 %) were positive for *Hepatozoon* sp., 13 (34.21 %) *L. chaquensis*, and 8 (22.86 %) *L. podicipinus*.

In Nhumirim, 72 animals were collected, of which 21 (29.16 %) were positive for *Hepatozoon* sp., 5 (16.67 %) *L. chaquensis*, and 16 (38 %) *L. podicipinus* (Table 1).

For PCR, statistical analysis showed that significant differences were not found when considering both frogs' collection site ( $\chi^2 = 0.003$ ,  $p = 0.958$ ), considering only *L. chaquensis* collection site ( $\chi^2 = 2.651$ ,  $p = 0.104$ ), and considering only *L. podicipinus* collection site ( $\chi^2 = 2.066$ ,  $p = 0.151$ ).

Considering the factor “used analysis techniques,” for both anurans species, statistically significant difference was found in the positivity between PCR and microscopy ( $\chi^2 = 23.995$ ,  $p < 0.001$ ).

### Morphological characterization of *Hepatozoon* sp.

The gamonts presented intraerythrocytic and extraerythrocytic characteristics. There were variable forms, some more rounded in with the nucleus taking the entire width of the gamont, and others with rounded ends, and sometimes the nucleus was dislocated towards the periphery, with nuclear chromatin ranging between fragmented and condensate. The gamonts showed format varying from “sausage” to the globular, with the coloration of basophilic cytoplasm and nucleus varying from blue-gray to pink. In most of the erythrocytes, the nucleus has been displaced to the periphery in the presence of the parasite (Figs. 1 and 2). Gamonts enlarging the host erythrocytes were not found. Means and standard deviations of morphometric parameters of gamonts are shown in Table 2. The morphometric analysis of gamonts showed that the coefficient of variation was relatively higher in *L. chaquensis* than in *L. podicipinus*.

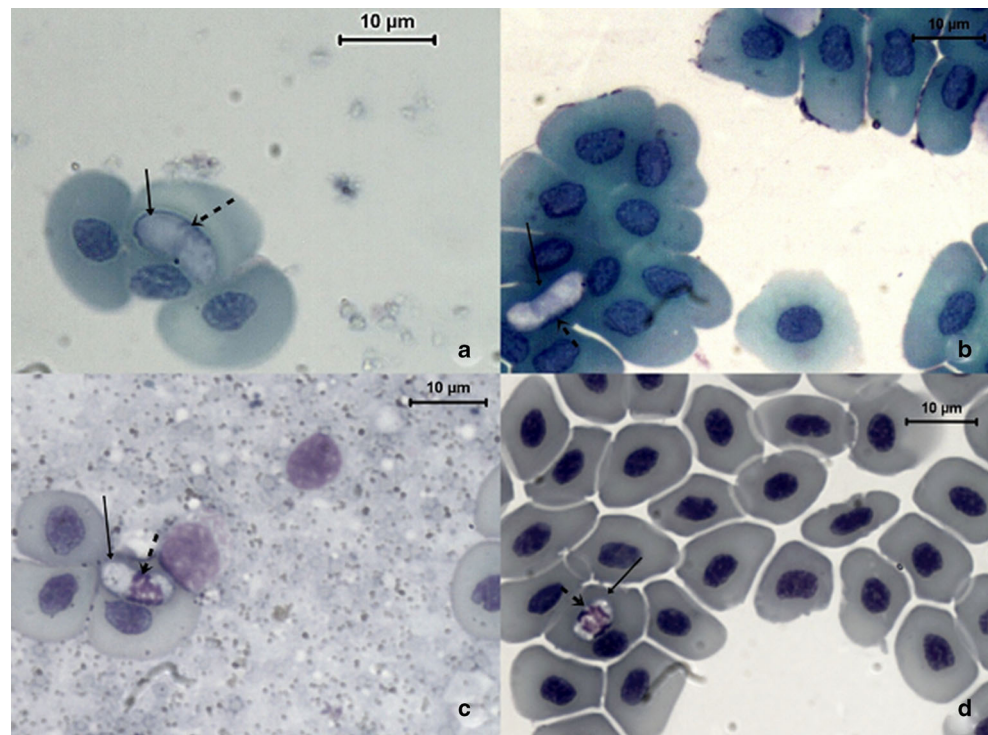
For 145 captured animals, a total of 133 organ imprints (spleen, liver, and kidney) were performed, 51 of

**Table 1** Positivity of *Hepatozoon* sp. in *Leptodactylus chaquensis* and *Leptodactylus podicipinus* collected in two regions of the Pantanal, Mato Grosso do Sul State, Brazil

Localities	<i>Leptodactylus chaquensis</i>			<i>Leptodactylus podicipinus</i>			Total
	Total number of hosts, <i>N</i>	Positive microscopy, <i>N</i> (%)	Positive PCR, <i>N</i> (%)	Total number of hosts, <i>N</i>	Positive microscopy, <i>N</i> (%)	Positive PCR, <i>N</i> (%)	
BEP	38	4 (10.5)	13 (34.2)	35	0	8 (22.9)	73
Nhumirim farm	30	4 (13.3)	5 (16.7)	42	2 (4.8)	16 (38.0)	72
Total	68	8 (11.76)	18 (26.50)	77	2 (2.6)	24 (31.2)	145



**Fig. 1** Intraerythrocytic gamonts of *Hepatozoon* sp. observed in *Leptodactylus chaquensis*. **a, b** Basophilic and condensed nucleus (caught at BEP); **c, d** acidophilic and fragmented nucleus (caught at Nhumirim farm). *Full arrow* indicates the gamont and *dashed* the nucleus. *Bar*, 10  $\mu$ m

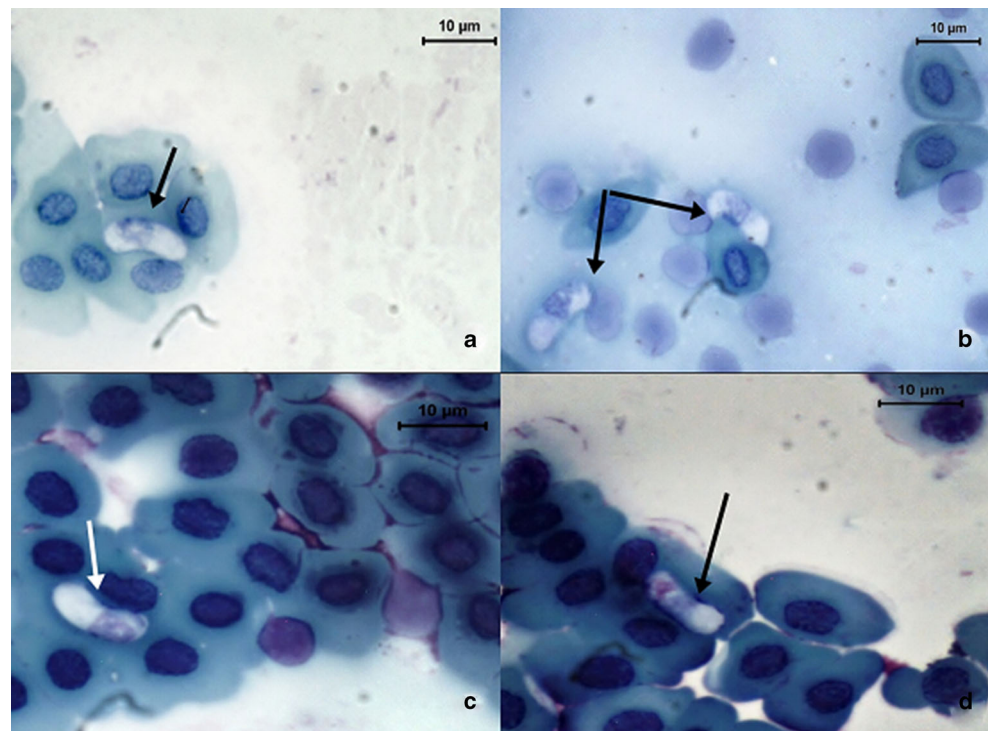


*L. chaquensis* and 82 of *L. podicipinus*. Schizonts were found in only four animals (3 %), three *L. chaquensis* (5.88 %), which presented three schizonts in the liver and ten in the spleen, and one in *L. podicipinus* (1.22 %), with one schizont in the liver. Four *L. chaquensis* (7.84 %) presented gamonts in the organs, six in the liver, one in the spleen, and one in the

kidney, whereas in *L. podicipinus*, gamonts were observed in two animals (2.44 %), one in the spleen and one in the kidney.

The schizonts were found in immature and mature phases of the cycle. Mature schizonts and merozoites were found in small number in these organs (Fig. 3a, b), where we could see mature schizonts with formed merozoites (Fig. 3a), and

**Fig. 2** Gamonts of *Hepatozoon* sp. observed in *Leptodactylus podicipinus* collected at Nhumirim farm. **a, c, d** Intraerythrocytic; **b** extraerythrocytic. The *arrow* indicates the gamont. *Bar*, 10  $\mu$ m



**Table 2** Morphometric parameters of *Hepatozoon* gamonts observed in blood smears of *Leptodactylus chaquensis* and *Leptodactylus podicipinus* collected in Pantanal region, Mato Grosso do Sul State, Brazil

Infected animals	Morphometric parameters					
	PA ( $\mu\text{m}^2$ )	PL ( $\mu\text{m}$ )	PW ( $\mu\text{m}$ )	NA ( $\mu\text{m}^2$ )	NL ( $\mu\text{m}$ )	NW ( $\mu\text{m}$ )
<i>Leptodactylus chaquensis</i> cv	35.05±10.0 (20.7–50.0), 28.4 %	9.7±2.7 (4.9–13.9), 28 %	4.5±0.7 (3.2–5.70), 14.9 %	16.25±5.3 (6.7–27.8), 32.4 %	4.7±1.4 (2.1–8.6), 29.55 %	4.2±0.75 (2.7–5.4), 17.9 %
<i>Leptodactylus podicipinus</i> cv	43.8±6.2 (30.15–49.2), 14.1 %	13.95±3.3 (7.1–17.2), 23.9 %	4.6±0.55 (4.0–5.8), 12 %	15.3±3.9 (9.25–21.05), 25.7 %	4.7±1.1 (2.3–6.0), 22.7 %	4.2±0.5 (3.65–5.3), 11 %

cv coefficient of variation, PA parasite area, PL parasite length, PW parasite width, NA nucleus area, NL nucleus length, NW nucleus width

schizonts with merozoites radially arranged around a residual body (Fig. 3b). The immature schizonts showed a “double line” around and contained coarse-looking material with no evident nucleus (Fig. 3c, d). As schizonts found were in different developmental stages, their measures were quite variable. In *L. chaquensis*, they measure  $297.23 \pm 169.1 \mu\text{m}^2$  (121.2–574.7) of area, whereas the unique schizont found in *L. podicipinus* has  $168.8 \mu\text{m}^2$  of area.

#### Molecular characterization

The use of HepF and HepR primers, in this study, was not able to amplify the sequences of *Hepatozoon* sp. for the size of the desired fragment of 625 bp, despite of many attempts changing the temperature and concentration of the PCR reaction products. Only the HEMOI and HEMOII oligonucleotides were efficient in amplification. The sequencing of all ten

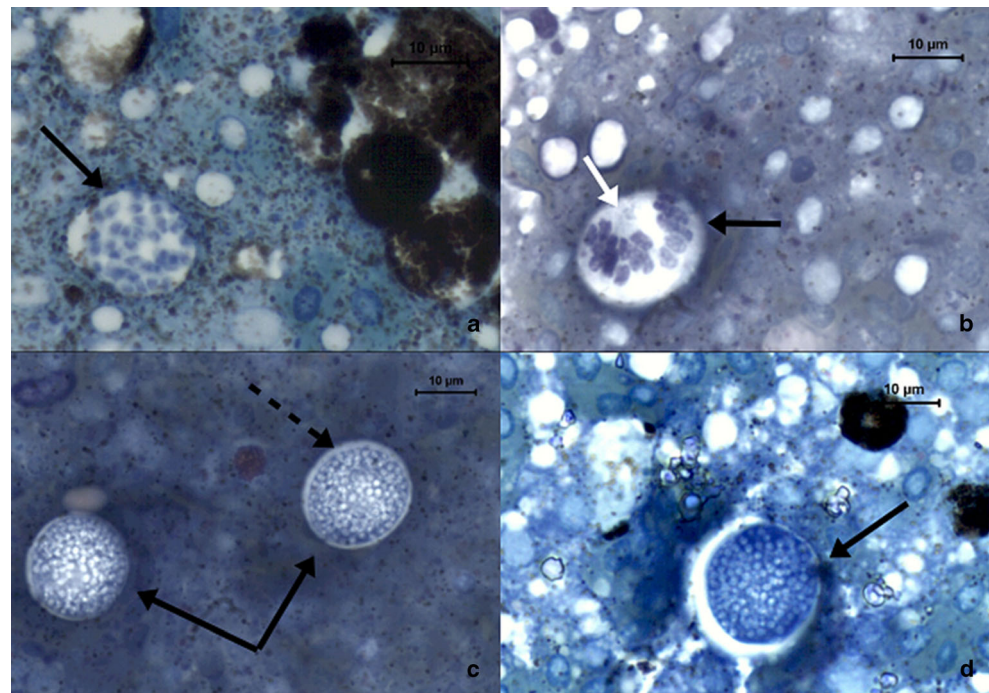
samples was conducted only for *L. chaquensis*. Due to lack of sufficient DNA in sample, the sequencing of *L. podicipinus* samples was not possible.

After their appropriate patterning, the use of oligonucleotides HEMOI and HEMOII (Perkins and Keller 2001) gave us products with expected size of approximately 900 bp.

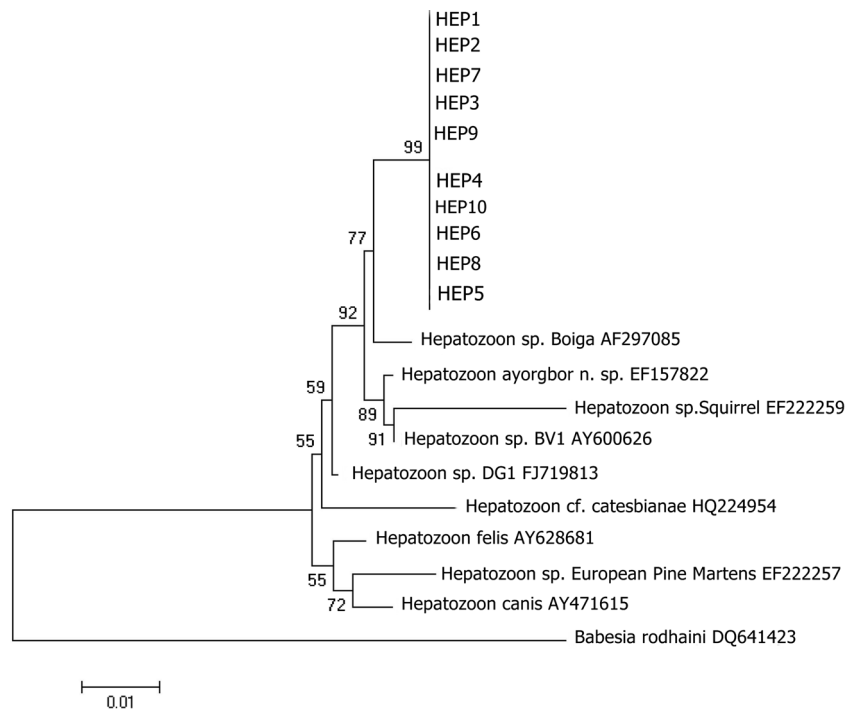
#### Phylogenetic analysis

All *Hepatozoon* sp. samples were amplified and sequenced (HEP1 to HEP10) generated sequences with 99 % of bootstrap. All samples (HEP1 to HEP10, GenBank sequence accession JX987775) were presented in the phylogenetic tree as a different branch from other haemogregarines described on other hosts. Samples identified in frogs showed more similarity to *Hepatozoon* sp. *Boiga* (unpublished) and are on a separate branch of other

**Fig. 3** Schizonts present in the spleen and liver. **a** *Leptodactylus podicipinus* (liver); **b, c** *Leptodactylus chaquensis* (spleen); **d** *Leptodactylus chaquensis* (liver). Black arrow indicates schizont; dashed arrow indicates double line; and white arrow indicates residual body. Bar, 10  $\mu\text{m}$



**Fig. 4** Neighbor-joining tree based on 18S RNA of ten samples of *Hepatozoon* sp. The numbers at nodes indicate bootstrap values for 1000 replicates. Scale indicates the evolutionary distance of 0.01 nucleotides per position in the sequence. Outgroup, *Babesia rodhaini*



sequences described as *Hepatozoon ayorgbor* n. sp., *Hepatozoon* sp. DG1, *Hepatozoon* sp. BV1, and in a more distant branch of *Hepatozoon* cf. *catesbianaes* (Fig. 4). In Table 3, there are the species of *Hepatozoon* with their intermediate hosts, used in the construction of the phylogenetic tree.

**Table 3** Species used in phylogenetic analysis including intermediate host, locality, and GenBank accession number

Intermediate hosts	Parasite	Locality	GenBank
<i>Boiga</i> sp.	<i>Hepatozoon</i> sp. Boiga	Australia	AF297085
<i>Python regius</i>	<i>Hepatozoon ayorgbor</i>	Africa	EF157822
<i>Sciurus vulgaris</i>	<i>Hepatozoon</i> sp. Squirrel	Spain	EF222259
<i>Clethrionomys glareolus</i>	<i>Hepatozoon</i> sp. BV1	Spain	AY600626
<i>Dromiciops gliroides</i>	<i>Hepatozoon</i> sp. DG1	Chile	FJ719813
<i>Rana catesbeiana</i>	<i>Hepatozoon</i> cf. <i>catesbianaes</i>	Canada	HQ224954
<i>Felis catus</i>	<i>Hepatozoon felis</i>	Spain	AY628681
<i>Martes martes</i>	<i>Hepatozoon</i> sp. European pine martens	Spain	EF222257
<i>Pseudalopex gymnocercus</i>	<i>Hepatozoon canis</i>	Brazil	AY471615
<i>Mus musculus</i>	<i>Babesia rodhaini</i>	–	DQ641423

## Discussion

The intraerythrocytic gamonts of *L. chaquensis* and *L. podicipinus* showed cytological and morphological changes that agree to the results of other studies performed worldwide (Fantham et al. 1942; Mohammed and Mansour 1966; Costa et al. 1973; Kim et al. 1998; Smith et al. 2000; Desser 2001; Leal et al. 2009). The gamonts of *L. chaquensis* are smaller than those reported by Costa et al. (1973), who obtained longer and wider gamonts. However, in relation to *L. podicipinus*, the size of gamonts was closest. We emphasize that the frogs studied by Costa et al. (1973) were *Leptodactylus latrans* and *Leptodactylus pentadactylus*.

The standard deviation between gamonts in two frogs species of our study showed that *L. chaquensis* gamonts were more varied in size and had lower size than those of *L. podicipinus*. A similar result was also observed in a previous study (Leal et al. 2009) with the same frog species. This variation may be attributable to the different developmental stages of the gamonts simultaneously present in the blood; this fact has been reported by Hull and Camin (1960) and Clark and Bradford (1969). This variability of gamont size was also observed by Smith et al. (1994) in *Nerodia sipedon sipedon*, parasitized with *Hepatozoon sipedon*, whose reptiles were exhibiting in your bloodstream many gamonts in the immature stage.

*Leptodactylus chaquensis* from BEP had more homogeneous forms of gamonts with predominance of medium size (Fig. 1a, b), and those caught in Nhumirim farm showed more variation, with small, medium, and large forms (Fig. 1c, d). It



was not possible to affirm that they represent the same *Hepatozoon* species, despite phylogenetic analysis.

Using microscopic analysis, it was not possible to observe gamonts in *L. podicipinus* collected in BEP. The gamonts observed in *L. podicipinus* caught at Nhumirim farm were homogeneous in their morphology and presented basophilic nucleus and cytoplasm.

For the collection site factor, the statistical analysis showed no significant difference in positivity of *L. chaquensis* and *L. podicipinus* infections with *Hepatozoon* sp., despite the ecological differences between the two sites, but there are other factors that were not taken into account in this study that may have influenced this positivity, such as the density of vectors or sexual difference of host (Bardsley and Harmsen 1973).

The scraping of blood smears was already used by some researchers, as Scopel et al. (2004) and Sykes et al. (2008), and demonstrated a lower sensitivity in comparison to whole-blood DNA extraction.

Statistical analysis of the false-positive results found in this work showed that there is no significant evidence that slide scraping is a factor that leads to the occurrence of false-negative results ( $\chi^2=0.325$ ,  $p=0.569$ ). In this case, the corrected value of  $\chi^2$  was used due to the presence of classes with frequencies lower than 5.

Anurans can be infected with many *Hepatozoon* species and can act also as parathenic hosts for others species (Smith 1996). Viana et al. (2012) proved that *L. chaquensis* is a parathenic host for *Hepatozoon caimani*. Cystozoites of *H. caimani* were identified in fresh liver impression smears of *L. chaquensis* after the ingestion with sporulated oocysts from laboratory-bred *Culex (Melanoconion)* mosquitoes. In our study, no cystozoites were found in organs of *L. chaquensis* or *L. podicipinus*, so we cannot incriminate the studied specimens as parathenic hosts.

Developmental stages of *Hepatozoon* sp. were found in anurans tissues. Immature and mature schizonts were observed in the spleen and the liver of *L. chaquensis* and in the liver of *L. podicipinus*. Mansour and Mohammed (1966), Levine and Nye (1977), and Dessler et al. (1995) also observed schizonts; however, these forms were only found in the liver. The mature forms of schizonts observed in this study are similar to those reported by Mohammed and Mansour (1966) and Levine and Nye (1977); however, the sizes reported by them are larger than we have found. Costa et al. (1973) reported mature schizonts relatively smaller than those of the present study.

The morphology and morphometry of our samples of *L. chaquensis* showed differences in gamonts and schizonts when compared to the literature; however, when analyzing the phylogenetic tree, we found a high degree of similarity among our samples, forming a separate branch. Phylogenetic analysis showed that the *Hepatozoon* species parasitizing *L. chaquensis* were identical, although these animals have been collected at points about 76 km apart one of other.

The use of HepF and HepR primers, in this study, was not able to amplify the desired sequences of *Hepatozoon* sp., agreeing to Moço et al. (2012), who used two pairs of primers (HEPF/HEPR and PIRO A1/PIRO B) to unsuccessfully detect *Hepatozoon* spp. in snakes. The success obtained with HEMOI and HEMOII oligonucleotides for amplification of fragments, agrees to O'Dwyer et al. (2013), who used added to HEMOI/HEMOII, the HepF300/Hep900 primers in combination, getting better results for amplifications of fragments of different isolated of *Hepatozoon* spp. in snakes.

It was also observed that the fragments (HEP1 to HEP10) are very close to those isolated of *Boiga irregularis*, an animal from Australia, whose fragments generated of haemogregarine, when compared with our samples, revealed to have little substitution between them. The study of Jakes et al. (2003), with *H. boigae* of these same reptile species, showed intraerythrocytic gamonts that did not distended the cell of host, but causing displacement of the erythrocyte nucleus, and the infected erythrocytes that stained more clearly than the nonparasitized cells. The gamonts observed in our study were not preferentially erythrocytic, and there was no whitening of the cell in the presence of the parasite. *Hepatozoon ayorgbor* n. sp., another species that is found in a branch near to our samples in the phylogenetic tree, presents some differences. In the study reported by Sloboda et al. (2007), the gamonts of *H. ayorgbor* n. sp. found in *Phyton regius* showed the body and the nucleus longer and narrower than those measured in our study. Sloboda et al. (2007) reported liver schizonts which were much smaller than those we found.

In comparison with *Hepatozoon cf. catesbiana*, a present species in *Rana catesbeiana*, our samples were positioned on a most distant branch and were quite different morphometrically. This morphological difference was also observed when comparing our results with those reported by Dessler et al. (1995). The gamonts found by them were bigger, showed a more elongated shape, basophilic weakly stained cytoplasm, compact and central nucleus. The gamonts we observed were not as elongated, and the nucleus were quite different, which were not only compact and central. Only the cytoplasm of gamonts was stained in a similar manner to that reported by the authors.

In this study, both analyzed species of anurans had a high positivity of infection with *Hepatozoon* sp. under the PCR analysis technique and there was no significant difference between the positivity for the two collection sites.

All comparisons done among the species of *Hepatozoon* of this study and those reported in the literature showed that our samples are different morphologically, morphometrically, and genetically, thereby demonstrating that the species of *Hepatozoon* in *L. chaquensis* observed in this study can represent a new species.

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