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PROTOZOOLOGY - ORIGINAL PAPER

Phylogenetic analysis of *Hepatozoon* spp. (Apicomplexa: Hepatozoidae) infecting *Philodryas patagoniensis* (Serpentes: Dipsadidae) in Uruguay

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Abstract

The purpose of this study was to perform a phylogenetic analysis of *Hepatozoon* spp. infecting *Philodryas patagoniensis* in Uruguay. Twenty-five road-killed specimens of *P. patagoniensis* from ten departments were obtained. Samples of blood and/or heart tissue were taken. Polymerase chain reaction (PCR) assay was carried out amplifying a specific target region of the 18S rRNA gene of *Hepatozoon* spp. Eighteen out of twenty-five samples were positive to *Hepatozoon* spp., which gave an overall prevalence of 72%. Phylogenetic analyses with the obtained sequences were carried out to determine the relationship with closely related species found in the region. The results revealed that samples were split into two clades with a high bootstrap support. Clade I was formed with *Hepatozoon* spp. sequences obtained in this study from *P. patagoniensis*, *Hepatozoon cuestensis* from *Crotalus durissus terrificus* and *Hepatozoon musa* from *Philodryas nattereri*, and *Hepatozoon* spp. retrieved from *Cerdocyon thous*, *Hemidactylus mabouia*, and *Phyllopezus pollicaris* from Brazil, respectively. Clade II was grouped with *Hepatozoon cevapii* and *Hepatozoon massardii*, both species described for *C. d. terrificus* from Brazil. This is the first report of *Hepatozoon* spp. in snakes from Uruguay.

Keywords *Hepatozoon* · *Philodryas patagoniensis* · Phylogenetic analysis · Uruguay

Introduction

The phylum Apicomplexa is a diverse group of obligatory parasites. It is estimated that currently, only 0.1% of the species from this phylum have been described (Morrison 2009). The genus *Hepatozoon* Miller, 1908 comprises more than 340 species of apicomplexan parasites closely related to piroplasmids and haemosporids, infecting a wide variety of amphibians, reptiles, birds, and mammals (Smith 1996; Baneth et al. 2003). The life cycle of *Hepatozoon* spp. involves sexual reproduction and sporogony in arthropods that serve both as vectors and as definitive hosts. To date, ticks (Ixodid and Argasid), mites (Acari), mosquitoes (Culicidae), sandflies (Phlebotominae), flies (Diptera), fleas (Siphonaptera), sucking lice (Anoplura), and triatomid bugs (Hemiptera) have been described as the definitive hosts of these parasites. Meanwhile, merogony followed by gametogony occur in vertebrate intermediate hosts. Arthropods acquire the infection by feeding on vertebrate blood cells infected with parasite gametocytes. Interestingly, vertebrates do not become infected through tick or insect bites as other apicomplexan such as Babesiidae family. Instead, the infected arthropods have to be ingested (Smith 1996). Species of *Hepatozoon* are commonly found parasitizing snakes, with several species described from snake hosts, such as *Hepatozoon cuestensis* O'Dwyer, Moço, Paduan, Spenassatto, Silva, and Ribolla, 2013, *Hepatozoon terzii* (Sambon and Seligman, 1907), and *Hepatozoon musa* Borges-Nojosa, Borges-Leite,

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Maia, Zanchi-Silva, da Rocha Braga, and Harris 2017 (Smith et al., 1999; Paperna and Lainson 2004; O'Dwyer et al., 2013; Borges-Nojosa et al., 2017).

Philodryas patagoniensis Girard, 1857 (Serpentes: Dipsadidae) is a South American snake with a wide distribution across Argentina, Bolivia, Brazil, and Paraguay. In Uruguay, *P. patagoniensis* is found throughout the country in a variety of habitats. It is considered a broadly-generalist snake, feeding on arthropods (spiders and ants), fishes, and amphibians, and also on other reptiles (such as saurians, amphisbaenians, ophidians, and even its same species), rodents and birds (Carreira and Maneyro 2013). To date, three species of *Hepatozoon* have been previously identified in snakes from *Philodryas* Wagler, 1830 genus. *Hepatozoon philodryasi* (Carini, 1910) from *P. patagoniensis* (reported as *P. schottii*), *Hepatozoon butantanensis* (Pessoa, 1928) from *Philodryas aestiva* (Duméril, Bibron and Duméril, 1854), and lastly, *H. musa* from *Philodryas nattereri* Steindachner, 1870; all the species were described in Brazil (Carini 1910; Pessoa 1928; Smith 1996; Borges-Nojosa et al., 2017).

The aim of this study was to molecularly characterize and compare the phylogenetic relationships among the *Hepatozoon* spp. detected in road-killed *P. patagoniensis* in Uruguay using a fragment of 18S rRNA gene.

Materials and methods

Sample collection

Twenty-five road-killed specimens of *P. patagoniensis* from ten departments of Uruguay were obtained (Fig. 1). The snakes were placed in individual plastic bags, identified, and kept with cool packs until arrival at the laboratory. Collection data are presented in Table 1. Species identification of collected snakes was done following Carreira and Maneyro (2013).

At the laboratory, blood was obtained directly from the heart, aorta, or vena cava. The samples were collected in 5 ml tubes with ethylenediaminetetraacetic acid (EDTA) and kept frozen at − 20 °C until DNA extraction. When the whole blood could not be extracted, heart tissue was retrieved. Blood smears from a single fresh road-killed specimen (V38) were prepared at the site of collection. Smears were air-dried, fixed with methanol, and stained with 1:9 May-Grünwald-Giemsa diluted in distilled water (Droguería Industrial Uruguaya, Uruguay). Compound microscope (Nikon E-200) was used for examination of slides under × 400 magnification.

DNA isolation, amplification, and sequencing

DNA was extracted from heart tissue and whole blood samples using the commercial kit PureLink™ Genomic DNA Mini Kit (Invitrogen, Germany), following the manufacturer's instructions. DNA of *Hepatozoon* sp. was amplified using PCR with the primers HEMO1 (5′-TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG-3′) and HEMO2 (5′-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3′). These primers amplify region of approximately 900 bp of 18S rRNA gene. Primers were designed by Perkins and Keller (2001), and PCR conditions were used following Harris et al. (2011). A second PCR was performed using the primer set HEP1mod (5′-CGC GAA ATT ACC CAA TTC TA - 3′) and HEP4 (5′-TAA GGT GCT GAA GGA GTC GTT TAT-3′) as described by Spolidorio et al. (2009). The second PCR cycle targeted a region of approximately 670 bp of the same gene. These primer pairs were selected because the fragments overlapped thus, a longer sequence (about 1300 bp) could be obtained. A positive (*Hepatozoon canis*) and negative (DNAse-free water) control were included in each reaction. PCR products were visualized under UV transillumination in a 1.5% agarose gel stained with GoodView™ Nucleic Acid Stain (Beijing SBS Genetech Co., LTd). The positive PCR products were purified using a PureLink™ Quick PCR Purification kit (Invitrogen, Germany) and sent to a commercial sequencing company (Macrogen Inc., Seoul, Korea).

Phylogenetic analysis

All the alignments and phylogenetic analysis were performed using MEGA 7 (Kumar et al. 2016). Each sequence was carefully checked and manual corrections were done when necessary. The two overlapping fragments of each sample were used to assemble a consensus sequence. The sequences obtained in this study were aligned with sequences retrieved from GenBank using MUSCLE algorithm. A phylogeny was constructed using the maximum likelihood (ML) algorithm. Best fitting evolutionary model for 18S rRNA gene was Tamura 3 parameters with gamma distribution. The support of the internal branching of the ML tree was assessed using 1000 bootstrap replicates. *Adelina grylli* Butaeva 1996 and *Adelina dimidiata* Schneider 1875 were used as outgroup.

Results

Morphology of the gamonts

The examination of the blood smears obtained from the V38 sample of *P. patagoniensis*, revealed the presence of *Hepatozoon* gamonts (Fig. 2). The gamonts showed a stout form, elongated shape with the ends broad and rounded. In some of the erythrocytes, a small elongation was evidenced, and the nucleus was displaced to a centrolateral position when the parasite was present. The mean measurements of the gamonts ($n = 6$) were the following: length 13.45 μ (11.60–15.00), width 4.83 μ (4.10–6.00), nucleus length 5.30 μ

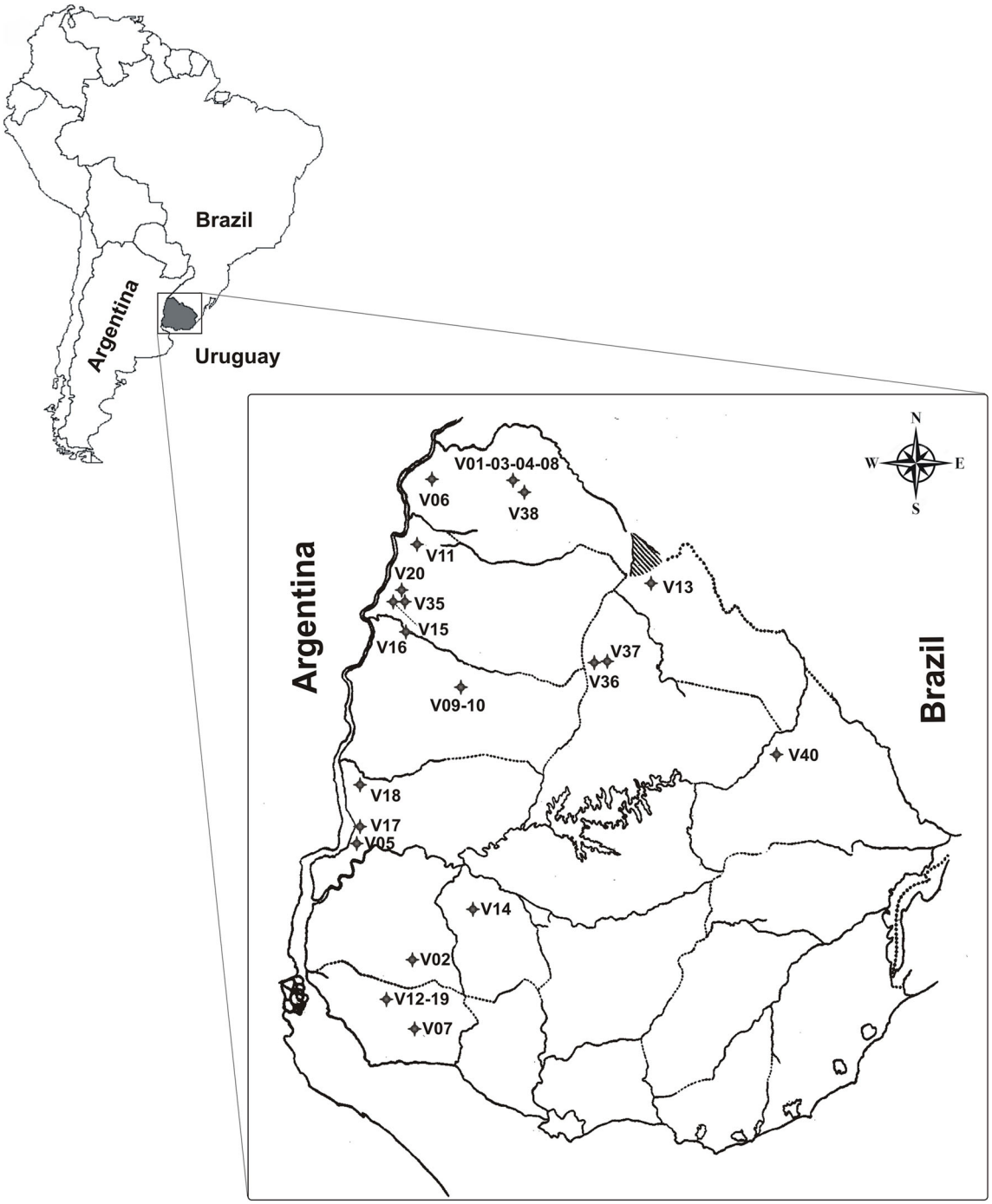

Fig. 1 Map showing road-killed *P. patagoniensis* collection sites

(4.40–6.00), nucleus width 3.53 μ (3.00–4.00). The measurements of the parasitized erythrocyte were the following: length 18.33 μ (17.50–20.00) and width 11.98 μ (9.60–14.00).

PCR and sequencing

A total of 18 out of 25 (72%) samples were positive for *Hepatozoon* spp. Fifteen samples were successfully amplified using both primer sets. The remaining three samples could only be amplified using a single primer set (Table 2). After assembling the overlapping fragments of each sample, the sequence lengths varied from 1199 to 1326 bp. Sequences obtained from the samples, V01, V02, V06, V10, V11, V14, V17, V19, V20, V21, V22, V35, V36, V37, and V38 have been submitted to GenBank (accession numbers MN003356 to MN003370, respectively).

Table 1 Data from the road-killed *P. patagoniensis* used in the study

Sample	Date	Site—department	Latitude, longitude
V01	April 7, 2012	Route 30, Colonia Artigas—Artigas	30°28′36″ S, 57°07′31″ W
V02	January 01, 2013	Route 55, km 57.200—Soriano	33°45′28″ S, 57°34′41″ W
V03	May 25, 2014	Route 30, Colonia Artigas—Artigas	30°28′36″ S, 57°07′31″ W
V04	September 20, 2013	Route 30, Colonia Artigas—Artigas	30°28′36″S, 57°07′31″W
V05	January 01, 2011	Route 24, Nuevo Berlin—Río Negro	33°02′20″ S, 58°01′10″ W
V06	February 01, 2015	Route 3, km 589—Artigas	30°33′05″ S, 57°40′07″ W
V07	April 02, 2014	Route 54, Barker—Colonia	34°15′55″ S, 57°27′47″ W
V08	August 30, 2015	Route 30, Colonia Artigas—Artigas	30°28′36″ S, 57°07′31″ W
V09	July 11, 2015	"El Relincho" farm—Paysandú	32°4′41″ S, 57°18′13″ W
V10	May 11, 2015	"El Relincho" farm—Paysandú	32°04′41″ S, 57°18′13″ W
V11	February 11, 2016	Route 3, km 536.9—Salto	31°00′87″ S, 57°42′12″ W
V12	November 16, 2015	Miguelete—Colonia	34°00′43″ S, 57°39′44″ W
V13	October 27, 2016	Route 30, km 242, Lunarejo—Rivera	31°10′5″ S, 55°50′59″ W
V14	December 29, 2015	Route 3, km 208—Flores	33°25′9″ S, 57°01′20″ W
V15	October 17, 2016	Barrio Artigas—Salto	31°22′59″ S, 57°53′15″ W
V16	September 30, 2015	Route 3, km 476—Paysandú	31°27′37″ S, 57°54′08″ W
V17	November 13, 2015	Route 24, km 17—Río Negro	33°04′22″ S, 58°02′50″ W
V18	March 27, 2016	Route 24, km 83.5—Río Negro	32°32′02″ S, 58°01′21″ W
V19	November 16, 2015	Miguelete—Colonia	34°00′43″ S, 57°38′50″ W
V20	February 11, 2016	Route 3, km 512—Salto	31°13′27″ S, 57°45′37″ W
V35	April 21, 2017	Route 31, km 18, San Antonio—Salto	31°22′57″ S, 57°44′59″ W
V36	December 22, 2016	Route 31, km 192—Tacuarembó	31°38′59″ S, 56°14′53″ W
V37	April 28, 2017	Route 31, km 190—Tacuarembó	31°38′48″ S, 56°15′55″ W
V38	November 03, 2017	Estación Cuaró—Artigas	30°36′47″ S, 56°54′17″ W
V40	February 16, 2019	Route 26, km 381—Cerro Largo	32°18′15″ S, 54°42′55″ W

Phylogenetic analysis

All the sequences obtained in this study were aligned with sequences retrieved from GenBank from various host species, and the final datasets contained a total of 45 sequences. Due to the difference in length of the available registered haemogregarines sequences, the tree was constructed using a fragment of 1250 bp. Therefore, three positive samples (V09, V15, and V18) could not be included in the tree due to the short length. These three sequences showed an identity of 99% with *H. musa* from *P. nattereri* and two species of *Hepatozoon* sp. from *Hemidactylus mabouia* Moreau De Jonnès, 1818 and *Rhinella schneideri* Werner, 1894, respectively (GenBank accession numbers, KX880079, KM234615, and MK508987). The phylogenetic analysis showed that the *Hepatozoon* spp. sequences obtained from *P. patagoniensis* of Uruguay are grouped in two monophyletic clades with a high bootstrap support (Fig. 3). Clade I was compound by 14 sequences obtained from *P. patagoniensis* (V01, V02, V10, V11, V14, V17, V19, V20, V21, V22, V35, V36, V37, and V38) and *H. musa* from *P. nattereri*, *H. cuestensis* from *Crotalus durissus terrificus* (Laurenti, 1768) and *Hepatozoon* spp. from the hosts *Cerdocyon thous* (Linnaeus, 1766), *Hem. mabouia*, and *Phyllopezus pollicaris* Spix, 1825. Clade II was constituted by V06 obtained from *P. patagoniensis* and sequences of *Hepatozoon massardii* O'Dwyer, Moço, Paduan, Spenassatto, Silva, and Ribolla, 2013, and *Hepatozoon cevapii* O'Dwyer, Moço, Paduan, Spenassatto, Silva, and Ribolla, 2013. Both species were described in *C. d. terrificus* from Brazil (Fig. 3).

Discussion

To date, there are only few published studies on *Hepatozoon* spp. from naturally infected *Philodryas* snakes (Carini 1910; O'Dwyer et al., 2003; Moço 2008; Borges-Nojosa et al., 2017). In this study, the observed prevalence of *Hepatozoon* spp. in *P. patagoniensis*, collected as roadkills, was 72%. The prevalence of *Hepatozoon* spp. reported in the current study was higher as compared with O'Dwyer et al. (2003) that reported a prevalence of 16% of *Hepatozoon* spp. in other members of the genus *Philodryas*. Furthermore, Moço (2008) screened nine *P. patagoniensis* without detecting the presence of *Hepatozoon* spp. These studies used only microscopy screening to determine prevalence of *Hepatozoon* spp.

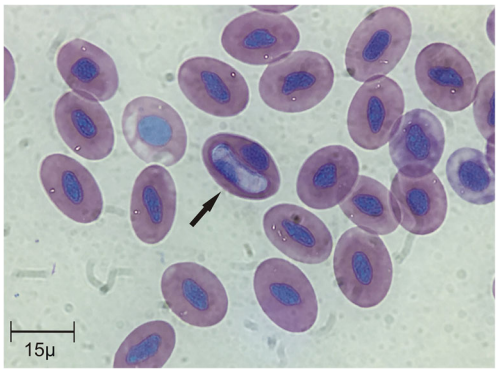

Fig. 2 Gamont of *Hepatozoon* sp. of a *P. patagoniensis* (May-Grünwald-Giemsa stain)

Borges-Nojosa et al. (2017) reported a low prevalence (14.3%) of species of *Hepatozoon* from seven *P. nattereri* in Northeastern Brazil.

Table 2 Samples of blood and hearth tissue obtained from *P. patagoniensis*. PCR results with the primers HEMO1-HEMO2 and HEP1mod-HEP4 are shown as positive (Pos.) and negative (Neg.)

Sample	Blood	Heart	HEMO1-HEMO2	HEP1mod-HEP4
V01	✓	✗	Pos.	Pos.
V02	✓	✗	Pos.	Pos.
V03	✓	✗	Neg.	Neg.
V04	✓	✗	Neg.	Neg.
V05	✓	✗	Neg.	Neg.
V06	✓	✗	Pos.	Pos.
V07	✓	✗	Neg.	Neg.
V08	✓	✗	Neg.	Neg.
V09	✓	✗	Neg.	Pos.
V10	✓	✗	Pos.	Pos.
V11	✗	✓	Pos.	Pos.
V12	✓	✗	Pos.	Pos.
V13	✓	✗	Neg.	Neg.
V14	✓	✗	Pos.	Pos.
V15	✗	✓	Pos.	Neg.
V16	✓	✗	Pos.	Pos.
V17	✓	✗	Pos.	Pos.
V18	✓	✗	Pos.	Neg.
V19	✓	✗	Pos.	Pos.
V20	✗	✓	Pos.	Pos.
V35	✓	✗	Pos.	Pos.
V36	✓	✓	Pos.	Pos.
V37	✓	✗	Pos.	Pos.
V38	✓	✓	Pos.	Pos.
V40	✓	✓	Neg.	Neg.

✓Sample obtained

✗Not obtained

One of the main drawbacks describing new species is the limited available information. For instance, in the past, most of the studies were based on morphological descriptions (Carini 1910, Pessoa, 1928). The risk of defining species based only on a single method (i.e., morphology of blood forms) was already recognized several decades ago (Ball 1958; Ball et al. 1967). However, as in O'Dwyer et al. (2013) and Borges-Nojosa et al. (2017), the present study combines morphological and molecular techniques for the characterization of *Hepatozoon* spp. from *P. patagoniensis*. Furthermore, in the present study, molecular techniques were shown to be a useful tool for characterization of *Hepatozoon* spp. from snake roadkills.

The morphometry of gamonts found on sample V38 were compatible with those found for *H. philodryasi*, the only species of *Hepatozoon* described from *P. patagoniensis* (Carini 1910; Moço et al. 2002). Furthermore, some of the analyzed gamonts showed an ovoid form, similar to *H. butantanensis* reported from *P. aestiva* (Pessoa, 1928). Although, the vermicular forms of *H. butantanensis* were larger as compared with the gamont forms reported in our study (up to 23 µ in length). On the other hand, the gamonts described as *H. musa*, found in *P. nattereri* were elongated (Borges-Nojosa et al. 2017), with a significant curvature at both ends and longer than those from our findings but similar to those described as the vermicular forms of *H. butantanensis*.

A problem in morphological-based studies is the polymorphism presented by the gamonts of certain species. For example, Smith (1996) mentioned three different forms of *Hepatozoon* gamonts in the blacktail rattlesnake *Crotalus molossus molossus* Baird and Girard, 1853. The same author reported the existence of immature and mature gamonts, and these developmental stages could be misdiagnosed as different species (Smith 1996). Much of this variability in the gamont structure could be attributed to the coexistence of different developmental stages in a single blood smear (O'Dwyer et al. 2013). According to Smith et al. (1994), snakes undergoing merogony may contain gamonts of different sizes and shapes representing immature or mature forms. As for snakes screened in South America, Moço et al. (2002) studied the morphology and morphometrics of gamonts such as length and surface as well as displacement of nucleus of parasitized erythrocytes of several species of *Hepatozoon*. The authors mentioned that *H. terzii*, *H. philodryasi* and a *Hepatozoon* sp. from *C. d. terrificus* could not be distinguished between. Some reports have demonstrated that the same *Hepatozoon* species could infect different hosts (Hull and Camin 1960; Pessoa et al. 1971). Besides, the gamonts of a same species of *Hepatozoon* could present slight morphologic changes and different cytopathological effects on the infected blood cells depending on the host (Ball et al. 1967; Pessoa et al. 1974). Some researchers (Pessoa et al. 1971, Smith 1996) argue that there is a possibility that some of the

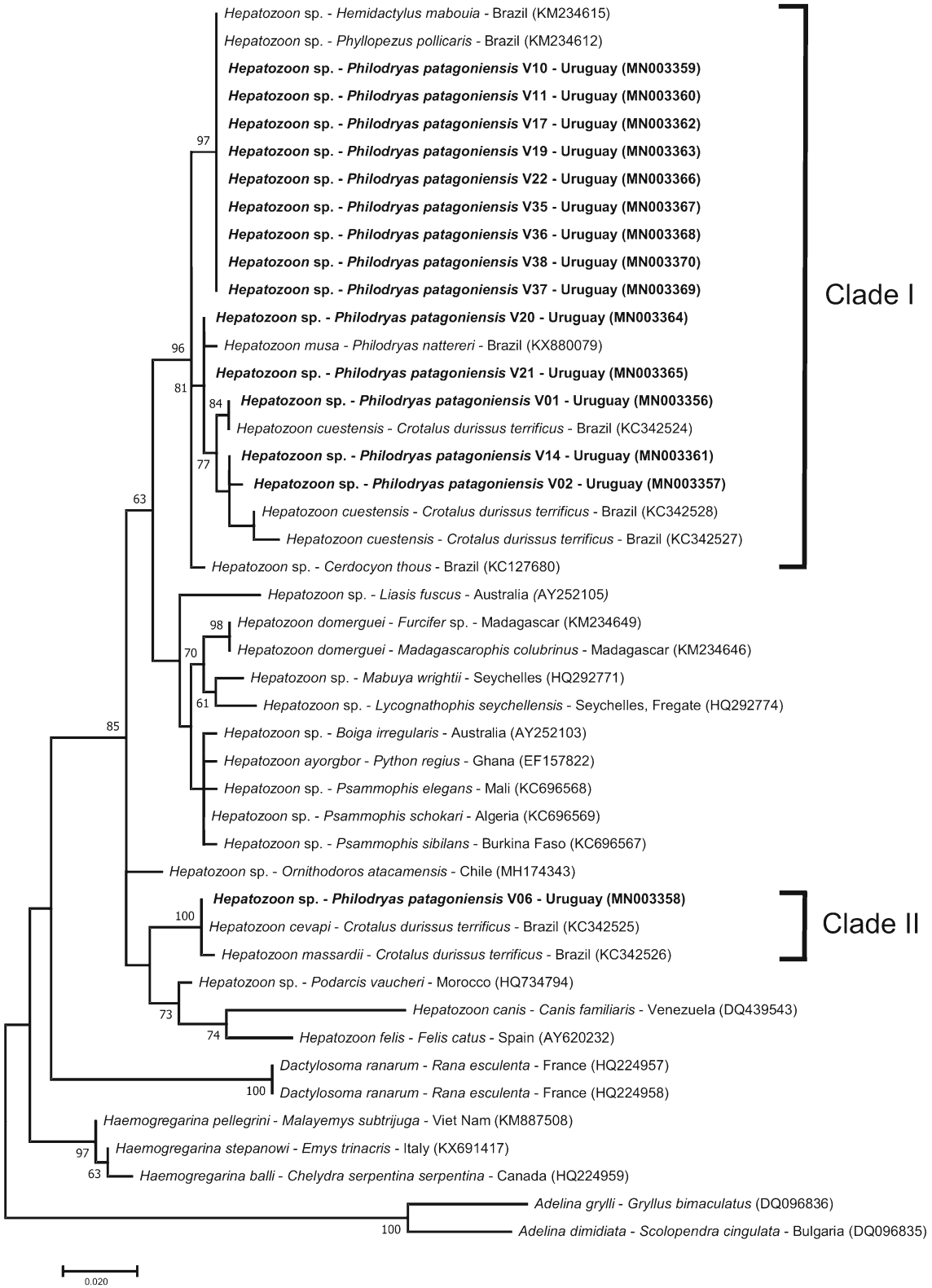

Fig. 3 Maximum likelihood tree constructed of *Hepatozoon* spp. partial 18S rRNA gene sequences. Numbers represent bootstrap support generated from 1000 replicates. The sequences obtained in this study were highlighted in bold. GenBank accession numbers are in brackets. *Adelina* spp. were included as outgroup. Brunch supporting value > 60 are shown

species of *Hepatozoon* described for terrestrial snakes are, in fact, the same species presenting pleomorphic blood forms that could vary depending on the vertebrate host.

The phylogenetic analysis performed using 18S rDNA sequences of *Hepatozoon* spp. from *P. patagoniensis* collected in Uruguay demonstrated that our samples are divided in two clades, with high bootstrap support (Fig. 3). All the obtained sequences from the present study (clade I), with the exception of one sample (V06), grouped with *H. cuestensis*, *H. musa* and *Hepatozoon* spp. found parasitising *P. pollicaris*, *Hem. mabouia*, and *C. thous*, respectively. According to our phylogenetic analysis, *H. musa* was shown to be closely related to *H. cuestensis*, and the other *Hepatozoon* sp. obtained in this study (Fig. 3). In contrast, sample V06 formed a separate clade (II) along with sequences of *H. cevapii* and *H. massardii* described from *C. d. terrificus* (O'Dwyer et al. 2013). For a proper species discrimination, further studies must be carried out with a larger number of samples from each *Hepatozoon* species. In our study, we showed the presence of two clades of *Hepatozoon* parasitizing *P. patagoniensis* from a restricted area of Uruguay. It is possible that some of the recently described species correspond to *H. philodryasi* or *H. butantanensis*, previously described from snakes of the genus *Philodryas*. Unfortunately, there are no molecular data available for *H. philodryasi* or *H. butantanensis* that could either confirm or deny this hypothesis. In agreement with Moço et al. (2002) and Maia et al., (2012), description of new *Hepatozoon* species must be carried out combining exhaustive morphological and molecular studies with longer fragments or full length of the 18S rRNA gene. This study showed that it is possible that some of the species described only using morphological features or short fragments of 18S rRNA gene could be the same species. Therefore, further studies on haemogregarines should include detailed morphological and extensive molecular data.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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