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PREVALENCE AND DIVERSITY OF *HEPATOZOON* IN NATIVE AND EXOTIC GECKOS FROM BRAZIL

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ABSTRACT: *Hepatozoon* is a genus of hemogregarines constituting the most widespread and common reptile hemoparasite. Although various molecular assessments of these parasites have been conducted in lizards from Africa and Europe, similar studies are needed for South American lizards. Through amplification and sequencing of fragments of the 18S rRNA gene, we assess the prevalence of *Hepatozoon* parasites in 230 geckos from South America, including the endemic species *Hemidactylus agrius*, *Hemidactylus brasilianus*, *Lygodactylus klugei*, *Phyllopezus pollicaris*, *Phyllopezus periosus*, and an exotic species, *Hemidactylus mabouia*. We found an overall low prevalence of *Hepatozoon* infection (7/230, 3%) with only 3 of the 6 host species infected with *Hepatozoon* (*Hemidactylus mabouia*, *P. pollicaris*, and *P. periosus*). Within the 7 infected host samples, 5 genetically distinct lineages of *Hepatozoon* parasites were identified, only 1 of which was similar to previously published haplotypes. Thus, although prevalence is low, genetically based diversity of *Hepatozoon* in geckos from South America is very high. Three of these lineages appear basal to 1 of the major clades of *Hepatozoon*, suggesting that this clade might have originated in South America, and thereby indicating a potential phylogeographic pattern that had not been previously identified. Future studies should assess the distribution and competence of invertebrate hosts in the regions analyzed, and *Hepatozoon* diversity in other less well-known regions of the world.

Apicomplexa, a disparate group of unicellular parasites, is one of the poorest studied of animal groups, with an estimate of less than 0.1% of species described (Morrison, 2009). Furthermore, studies are biased towards certain groups with particular importance to humans, such as *Plasmodium* (Gardner et al., 2002). There is a similar bias concerning hosts, with many studies of infections in birds (Knowles et al., 2010) and mammals (Claser et al., 2014), but fewer in other vertebrate groups (Perkins and Austin, 2009) such as the reptiles. Yet for some Apicomplexa, including *Plasmodium* Marchiafava and Celli, 1885 and the common and widespread *Hepatozoon* Miller, 1908, more species have been described from reptiles relative to other tetrapods (Smith, 1996).

Molecular techniques have the potential to revolutionize parasite monitoring (Stensvold and Nielsen, 2012). In particular, screening can be used to assess the distribution of known parasite lineages in different geographic regions and host species, to improve knowledge of host specificity and to determine biogeographical patterns (Perkins and Schall, 2002). These might be particularly important in reptiles, because the high diversity of parasite species in these hosts has been linked to their greater phyletic age relative to birds and mammals, and their typically reduced vagility (Telford, 2009). Such factors also mean that biogeographical patterns may be more distinct. However, most of these studies of *Hepatozoon* in reptiles have been geographically limited to Europe and North Africa. To better understand biogeographic patterns it is necessary to assess lineages of parasites in other hosts from different regions. For instance, the 2 *Hepatozoon* species described from canids are geographically disjunct, with *Hemidactylus americanum* Vincent-Johnson et al., 1997 in the Americas, and *Hemidactylus canis* (James, 1905) in Africa, Asia, and Europe (Baneth et al., 2003).

More than 300 species of the genus *Hepatozoon* have been described, mostly based on occurrence of gamonts in the erythrocytes, or occasionally the leucocytes (Herbert et al., 2010), of vertebrate intermediate hosts, and particularly reptiles, in which they are the most frequently reported hemogregarines (Smith, 1996). Molecular screening with the use of primers for part of the 18S rRNA gene have identified *Hepatozoon* in various different vertebrate host samples, including entirely new intermediate host groups such as bats (Pinto et al., 2013) and caecilians (Harris et al., 2014), and additional host taxa within birds (Biedrzycka et al., 2013), and reptiles (Maia et al., 2012b; Tomé et al., 2014). Phylogenetic analyses of these sequences have revealed unexpected aspects of their evolutionary history, such as a lack of coevolutionary pattern with the vertebrate host (Harris et al., 2012) and other aspects of transmission, including an apparent trophic link in both saurophagous snakes (Tomé et al., 2014) and possibly carnivores and their prey (Maia et al., 2014a). In addition, studies have suggested that coevolution may be occurring rather with the definitive (invertebrate) host (Barta et al., 2012). However, knowledge concerning parasite developmental stages within the invertebrate final hosts remains poor.

The aim of this study was to assess the prevalence and genetic diversity of *Hepatozoon* in geckos from Brazil (Gekkonidae: *Hemidactylus* Oken, 1817 and *Lygodactylus* Gray, 1864; Phyllodactylidae: *Phyllopezus* Peters, 1877) and place these parasites in a phylogenetic framework to investigate possible biogeographical patterns. Geckos are a good initial approach for this because data are available for other geckos from other regions, such as North Africa (Maia et al., 2011) and islands of the Indian Ocean (Harris et al., 2011), and from other lizards from southern Europe (Maia et al., 2012b). Currently, Brazilian hosts 3 native species of *Hemidactylus* (*Hemidactylus agrius* Vanzolini, 1978, *Hemidactylus brasilianus* Amaral, 1935, and *Hemidactylus palaichthus* Kluge, 1969) and 1 exotic species (*Hemidactylus mabouia* Moreau de Jonnès, 1818), 2 native species of *Lygodactylus* (*Lygodactylus klugei* (Smith, Martin and Swain, 1977) and *Lygodactylus wetzeli* (Smith, Martin, and Swain, 1977)), and 3 native *Phyllopezus* (*Phyllopezus lutzae* (Loveridge, 1941), *Phyllopezus periosus* (Rodrigues, 1986) and *Phyllopezus pollicaris* (Spix, 1825)) (Bérnils

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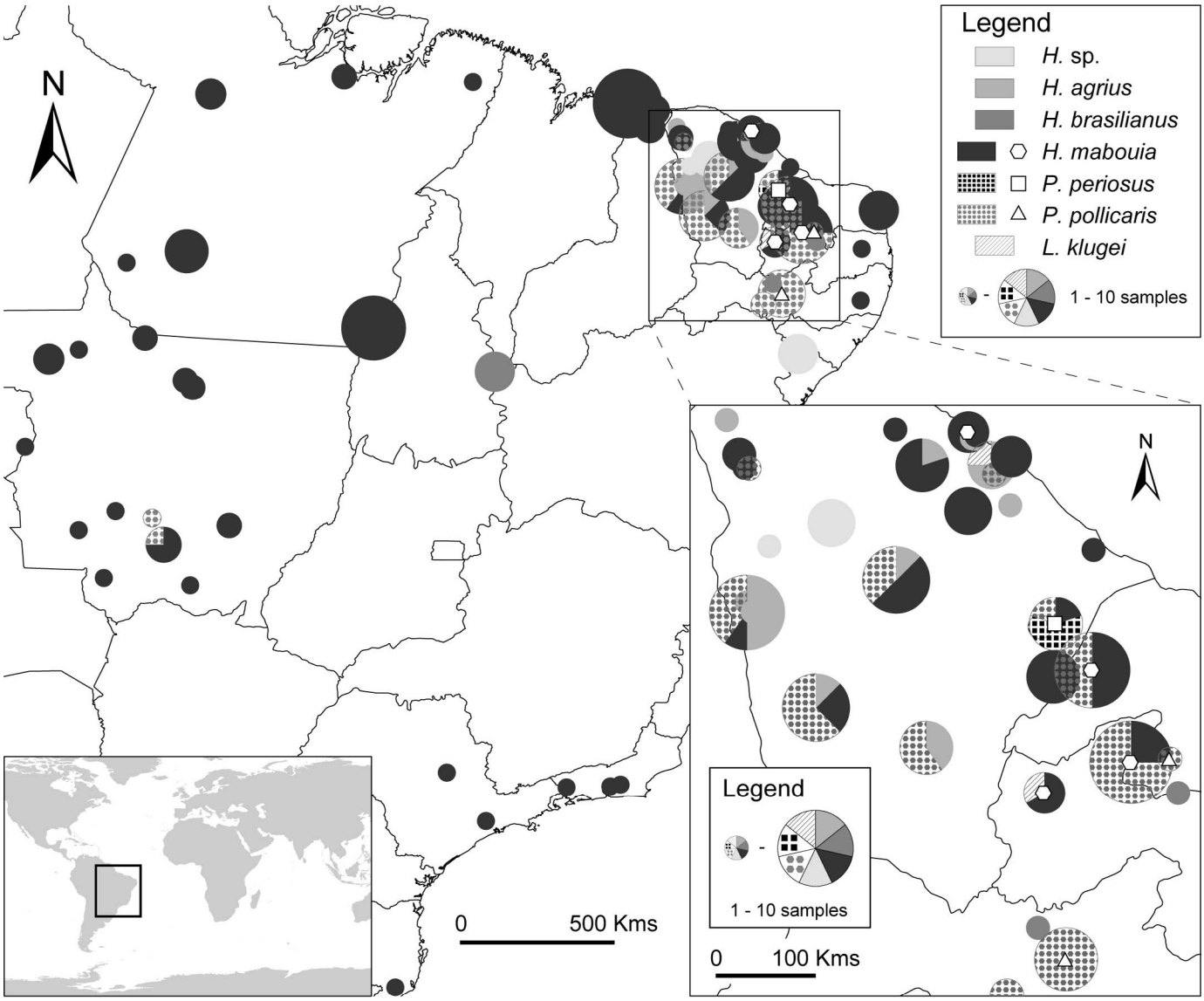

FIGURE 1. Map of Brazil indicating the location of the samples analyzed in this study. Tones of gray indicate the different species, and the sizes of the circles indicate the number of individuals analyzed per location. White geometric shapes inside the circles indicate the positive samples for each host species and location.

and Costa, 2012). All native species are endemic to South America (Gamble et al., 2007, 2008). By screening both native and exotic host species, we also assess if parasites in exotic hosts are similar to those from their point of origin. *Podarcis* Wagler, 1830 wall lizards in North America, introduced from Italy, are generally parasite depauperate (Burke et al., 2007). Again, by screening exotic *Hemidactylus* we can assess whether or not there is an entire lack of infection or a lack of blood parasite diversity in exotic populations of these geckos. Recently 3 new species of *Hepatozoon* from rattlesnakes *Crotalus durissus terrificus* (Laurenti, 1768) were described from Brazil, with the use of both morphological and molecular data (O'Dwyer et al., 2013). Because *Hepatozoon* from snakes and lizards are very similar based on 18S rRNA sequences in North Africa (Tomé et al., 2012, 2013), we can also assess if the same pattern occurs in South America. Finally, the primers chosen for use in this study are known to occasionally amplify other Apicomplexan and related parasites (Harris et al., 2012). By sequencing all positive host samples, we also aim to determine if other parasite genera are detected in these hosts (Maia et al., 2012a).

MATERIALS AND METHODS

Sample collection

Tissue samples from gecko specimens collected from across Brazil (see Fig. 1) and preserved for molecular analysis (tail tips or liver tissue, stored in 96% ethanol) were used to detect the presence of parasites. Blood smears for these samples were not available. In all cases, animals were collected and identified, and the GPS coordinates registered. Some specimens were released at the place of capture, and others were fixed with 10% formalin, preserved in 70% alcohol, and placed in the collections of the University Federal do Ceará (CHUFC), collection license number 10893-1/IBAMA number 472138. Other specimens were obtained from museum collections (AAGARDA—Collection of Herpetology of Dr. Adrian Garda; CHUNB—Collection of Herpetology of

TABLE I. Family and species of lizards included in this study. For each species, the total number of individuals analyzed and the number of infected samples with *Hepatozoon* are given, as tested through PCR amplification and confirmed by DNA sequencing. Observed prevalence is calculated with the use of the total number of gecko hosts analyzed and the total number of infected individuals. The numbers that correspond to samples from muscle and liver, respectively, are given inside parentheses.

Family	Species	Total analyzed	Total infected	Observed prevalence (%)
Gekkonidae	*Hemidactylus agrius*	21 (11,10)	0*	0
	Hemidactylus brasilianus	7 (4,3)	0	0
	Hemidactylus mabouia	142 (111,31)	4 (4,0)	2.8
	Hemidactylus sp.	10 (0,10)	0	0
	Lygodactylus klugei	2 (1,1)	0	0
Phyllodactylidae	*Phyllopezus periosus*	4 (3,1)	1 (1,0)	25.0
	Phyllopezus pollicaris	44 (37,7)	2 (1,1)	4.6
Total		230 (167,63)	7 (6,1)	3.0%

* Another sequence (KM234611), obtained from a liver sample (CHUFPB02513), appeared to be related to the genus *Eimeria* and was not analyzed further.

UnB; CHUFPB—Collection of Herpetology of UFPB; MNRJ—Museu Nacional of Rio de Janeiro; MPEG—Museu Paraense Emílio Goeldi; MTR—Collection of Tissues of Dr. Miguel Trefaut Rodrigues; MZUSP—Museu de Zoologia da USP; UERJ—Collection of Herpetology of UERJ; UFBA—Collection of Herpetology of UFBA; and UFMT—Collection of Herpetology of UFMT), in which case either muscle or liver samples were used. A total of 230 tissue samples were collected, from 3 genera (*Hemidactylus*, *Phyllopezus*, and *Lygodactylus*) and 6 species (see Table I).

DNA extraction, amplification, and sequencing

DNA was extracted from tissue with the use of standard High Salt methods (Sambrook et al., 1989). Detection of *Hepatozoon* parasites was initially made with the use of PCR reactions with the primers HepF300 and HepR900, targeting part of the 18S rRNA region (Ujvari et al., 2004). These are known to amplify various blood parasites (Harris et al., 2012). Subsequently, positive samples were further tested with the primers HEMO1 and HEMO2 that target another part of the 18S rRNA region (Perkins and Keller, 2001). These primers are less efficient; that is, some positive samples with the Hep primers do not amplify (Maia et al., 2012b; O'Dwyer et al., 2013), but when successful, allow a longer fragment of DNA to be analyzed in conjunction with the fragment from the Hep primers. Briefly, PCR cycling for the Hep primers consisted of 94 C for 30 sec, 60 C for 30 sec, 72 C for 1 min (35 cycles), whereas for Hemo primers the annealing temperature was 48 C (Harris et al., 2011). Negative and known *Hepatozoon* positive controls were run with each reaction. PCR products were analyzed by electrophoresis in 2% agarose and visualized by Gel Red staining and UV transillumination. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen, Inc.). All sequences were performed in both directions.

Phylogenetic analysis

Sequences for 7 positive samples obtained from the Hep primers were combined with *Hepatozoon* sequences retrieved from GenBank, and aligned with the use of ClustalW software implemented in the program BioEdit (Hall, 1999). The final data set contained 54 sequences, 540 bp in length, except KC127690, which was missing the first 52 base pairs (bp), and the 5 sequences from O'Dwyer et al. (2013), KC342524-8, which were missing the last 90 bp. All analyses were performed on this data set, although 3 samples were also sequenced with the Hemo primers, and for these the longer consensus sequences were obtained (KM234614, KM234615, and KM234617). All sequences were submitted to GenBank (accession numbers KM234611–KM234618).

Maximum-likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to estimate evolutionary relationships. Support for nodes was estimated with the use of the bootstrap technique (Felsenstein, 1985) with 1,000 replicates. The AIC criterion conducted in jModeltest 0.1.1 (Posada, 2008) was used to choose the best model of evolution and the parameters employed (i.e., TVM+G). Bayesian analysis was also implemented with the use of Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 1×10^6 generations, saving 1 tree each 1,000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree, in which frequency of any particular clade represents the posterior probability (Huelsenbeck and Ronquist, 2001). Following Barta et al. (2012), *Haemogregarina balli* Paterson and Desser, 1976 and *Dactylosoma ranarum* Lankester, 1892 were used as outgroups for rooting the phylogenetic tree.

RESULTS

Of the 230 samples of geckos screened, 8 were positive with the Hep primers. Seven of these were identified as *Hepatozoon* (3% prevalence, Table I), whereas the other sequence was identified as an unknown Eimeriorinid with the use of the BLAST comparison algorithm on GenBank. For *Hepatozoon*, 4 of these were from *H. mabouia* (GenBank accession numbers KM234615, KM234616, KM234617, and KM234618), 1 from *P. periosus* (KM234614) and 2 from *P. pollicaris* (KM234612 and KM234613). With the use of the Hemo primers, 3 of these could be successfully amplified, but in order to include all new sequences only the shorter fragment was used for the phylogenetic analysis (Fig. 2). *Hepatozoon* positives were found in the northeastern part of the sampling region (Fig. 1). The other, non-*Hepatozoon*, sequence (KM234611, sample CHUFPB02513 from an *H. agrius* host) appeared to be related to the genera *Caryospora* Léger, 1904 (KC696572 and AF060976, 95% similarity) and *Eimeria* Schneider, 1875 (AF307877 and AB544336, 95% and 94% similarity, respectively). These are other Apicomplexan parasites, but because the genus *Eimeria* is paraphyletic and includes other parasites (Megía-Palma et al., 2013, 2014), this was not analyzed further.

Of the 7 positives, 5 new *Hepatozoon* haplotypes were retrieved. Our estimate of phylogeny indicates that these new haplotypes form 5 distinct lineages (Fig. 2). Two individuals of *H. mabouia* were found to be infected with *Hepatozoon* similar to those already identified in rodents and geckos, most closely to rodents from Chile (Fig. 2, labeled A). The other lineages were all highly divergent from previously published lineages, but all formed part of the same major clade within *Hepatozoon*. Another lineage (Fig. 2, labeled B) is related to 1 of the lineages of *Hepatozoon* from rattlesnakes (KC342524, KC342527, and KC342528) and then to 1 in which South American foxes as hosts (KC127680). The

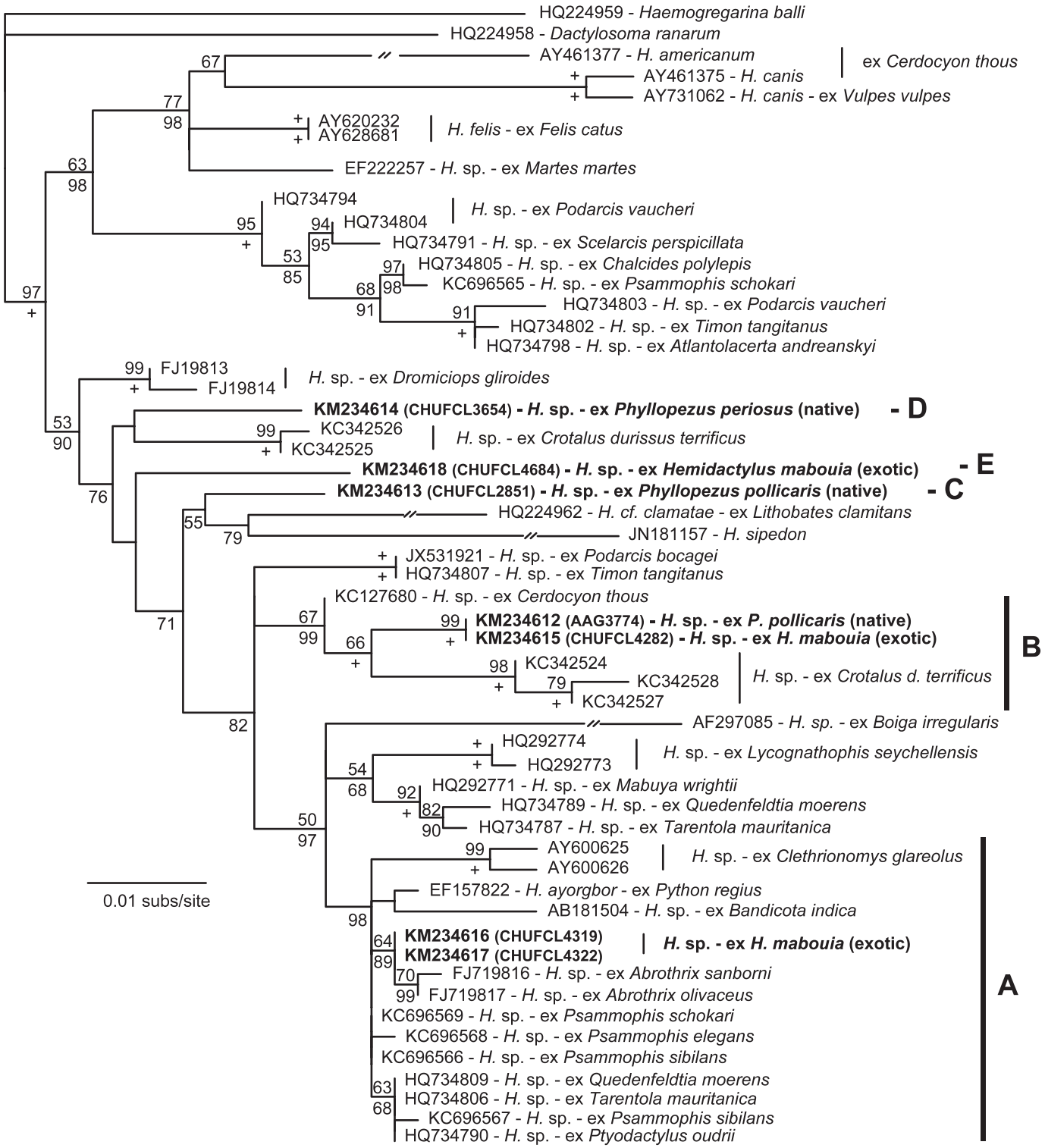

FIGURE 2. Estimate of relationships based on a ML analysis for 18S rRNA gene fragments of *Hepatozoon*. Bootstrap values for ML are given above relevant nodes and Bayesian posterior probabilities are given below them. When values were 100%, this is indicated with a +. The sequences indicated in bold represent those from this study and original sample codes given inside parentheses.

relationship of the other lineage (Fig. 2, labeled E) is unsupported, but is unlike any lineage previously identified in geckos from North Africa. Two individuals of the endemic South American genus *Phyllopezus* were positive for *Hepatozoon* (2/49, 4.1%), and both represent new and genetically distinct lineages (Fig. 2, labeled C and D). The relationships of 1 lineage (C) are poorly supported, although it may be more closely related to a lineage known from amphibians (HQ224962 and JN181157). The other

lineage (D) from *Phyllopezus*, along with the lineage of *Hepatozoon* from South American marsupials (FJ719813 and FJ719814), a lineage from rattlesnakes from Brazil, and 1 lineage (E) from *H. mabouia*, all form basal branches within 1 of the major clades within *Hepatozoon*.

DISCUSSION

Our results show an unexpected diversity of *Hepatozoon* lineages within geckos from South America. In comparison, for example, Maia et al. (2011) examined 460 lizards from North Africa, and recovered only 4 lineages, 3 of which were already known. In Brazil, with fewer host samples screened ($n = 230$), 5 lineages were found, and only 1 of these had been previously identified. This demonstrates the value of screening samples from other geographical regions to fully appreciate the diversity within *Hepatozoon*. Although the samples came from 2 tissue sources (muscle and liver), because they were specimens collected for different reasons, all the positive samples were from muscle tissues except 1 liver tissue sample from a *P. pollicaris*. However, it is known that *Hepatozoon* parasites can be detected from blood and tissue samples (Maia et al., 2014b), and although comparisons of prevalence between studies with the use of different tissue sources is complex, our results show that even museum specimens can be useful sources of information regarding these parasites.

Concerning the exotic species *H. mabouia*, it clearly can host a variety of *Hepatozoon* species, because it was host to multiple different parasite lineages. However, these may be lineages from South America rather than introduced with *H. mabouia*. The single lineage that was previously known is most closely related to haplotypes identified in rodents from Chile. Another lineage is related to 1 of the lineages from rattlesnakes and then to 1 from South American foxes, although this latter lineage is presumed to be from prey items, as it is clearly genetically not related to *Hepatozoon canis* or *Hepatozoon americanum* (Almeida et al., 2013). The 2 individuals of the endemic South American genus *Phyllopezus* that were infected with *Hepatozoon* represent new and genetically distinct lineages. In general they form the basal lineages within 1 of the *Hepatozoon* major clades, along with parasites from South American marsupials and rattlesnakes. Therefore it is currently parsimonious to assume that this major clade first diverged within South America, later spreading to other regions. However, much more data from other biogeographical regions, particularly Asia and Southern Africa, would be needed to confirm this.

This study is similar to other studies regarding the overall estimate of relationships between different *Hepatozoon* lineages. Two major groups can be identified, 1 predominantly composing *Hepatozoon* from carnivores, and a clade of *Hepatozoon* from North African and western Mediterranean reptiles. The other group includes all the new lineages identified here, plus many *Hepatozoon* from lizards, rodents, and snakes. However, the picture is complicated by apparent infection of intermediate hosts through trophic pathways. Snakes appear to be often infected by the same lineages of *Hepatozoon* as their lizard prey (Tomé et al., 2014), and recently some canids have also been shown to be infected by lineages other than *H. canis* and *H. americanum*, but similar to those found in possible prey items (Almeida et al., 2013; Maia et al., 2014a). On the other hand, the lineages from rattlesnakes from Brazil, recently identified as new species (O'Dwyer et al., 2013), are clearly genetically distinct from those reported in geckos in this study. However, prevalence in this study was low and more data are needed to confirm this. It is apparent that assessment of final hosts, particularly ticks, mites, mosquitoes, and biting flies will be necessary to resolve the issue of *Hepatozoon* diversity and how specific they are to intermediate hosts fully. There does seem to be very little coevolutionary signal, however, particularly in geckos that can host an array of *Hepatozoon* lineages from across the phylogeny of these parasites. The increasing data collected from various geographical regions seems to support that a revision of *Hepatozoon* taxonomy is needed (Smith and Desser, 1997). However, this will only be advisable after analyzing additional, and faster-evolving, genes of these parasites. Furthermore, assessing additional genes will clarify whether paralogous rRNA genes are potentially obscuring the phylogenetic picture. This seems unlikely, given that various clades are taxonomically or geographically coherent, but is always a potential problem with analyses based solely on multicopy genes such as the 18S rRNA.

To conclude, 5 different genetic lineages of *Hepatozoon* and 1 unknown Eimeriorinid were identified in geckos from Brazil. This further shows the genetic diversity of parasites within *Hepatozoon*, and particularly those from reptile intermediate hosts. The exotic species *H. mabouia* was host to multiple different parasite lineages. A tentative biogeographical pattern is emerging, with South America including the basal lineages within 1 of the major *Hepatozoon* clades. However, further screening of other regions would be needed to confirm this.

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