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MOLECULAR SCREENING OF *PLASMODIUM* (HAEMOSPORIDIA: PLASMODIIDAE) PARASITES FROM REPTILES IN BRAZIL

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ABSTRACT

Hemosporidians are a monophyletic group of protozoan parasites infecting all terrestrial vertebrate orders. Although *Plasmodium* is the most studied genus within the Haemosporidia, this research effort is heavily biased toward mammal and bird hosts. We screened 205 specimens of at least 18 reptile species from Brazil using a partial mitochondrial cytochrome b gene marker. Positive samples were sequenced and included in a phylogenetic assessment. Four positive PCR products matched others identified as *Plasmodium* using BLAST from 3 different host species, *Ameiva ameiva*, *Tropidurus hispidus*, and *Hemidactylus mabouia*. Recovery of similar haplotypes in the native *T. hispidus* and exotic *H. mabouia* (99.9%) indicate potential host-switching.

The Haemosporidia are a diverse order of vector-transmitted, intraerythrocytic parasitic alveolates. Plasmodiidae is the most studied family within the Haemosporidia, unsurprisingly, given that malaria is one of the most important infectious diseases in the world in terms of human economic costs and deaths. However, historically, research effort has been concentrated on certain host groups, such as mammals and birds, while information regarding parasite diversity, distribution, and virulence in reptiles is far from complete. This has considerable implications since more species of *Plasmodium* have been described from reptiles—over 100 based on morphological characters (Telford, 2009)—than in either mammals or birds. Molecular phylogenetic relationships indicate that hemosporidian parasites from reptiles form several diverse and unrelated lineages (e.g., Pineda-Catalan et al., 2013; Maia et al., 2016; Galen et al., 2018b). Therefore, without additional sampling across reptiles, any overall estimate of relationships is incomplete. Several *Plasmodium* subgenera infecting reptiles have been proposed (Telford, 1988), but with genetic data available for only a few species, it is not clear if these are monophyletic units or how they are related. What is clear is that within *Plasmodium* there is considerable taxonomic conflict (Galen et al., 2018a).

As well as being necessary to assess phylogenetic relationships, the use of molecular markers has revolutionized understanding of the diversity of malarial parasites, with many examples of cryptic species being recovered (e.g., Perkins, 2000). Again, however, while vast screening efforts have helped clarify the situation in, for example, birds (e.g., Bensch et al., 2000), the number of studies concerning reptiles is still minimal. Matta et al. (2018) recently screened reptiles in Colombia, redescribing *Plasmodium kentropyxi* and *Plasmodium carmelinoi*. Other than this, there are almost no molecular data available from *Plasmodium* infecting reptiles from South America, despite the vast array of potential hosts in a region that includes known biodiversity hotspots such as the Amazon and Atlantic forest systems of Brazil.

The aim of this study was to opportunistically screen reptiles from Brazil, which had generally been sampled as part of phylogeographic studies of the hosts themselves, and to assess the diversity of the parasites in a phylogenetic framework. By screening hosts in this way, infection rates within species can potentially be examined.

Tissue samples from 205 individuals representing at least 18 reptile species were collected from across Ceara State, Brazil and preserved for molecular analysis by storage in 96% ethanol (Table I; and Suppl. Material). These specimens were collected for other original purposes, with some sampled and released while others were fixed in 10% formalin, preserved in 70% alcohol, and maintained in the collection of the University Federal do Ceara (CHUFC). The molecular approach followed standard procedures used in other similar studies (e.g., Matta et al., 2018). DNA was extracted from the different tissues using high salt procedures, which are as effective as commercial kits for extracting parasite DNA (Maia et al., 2014). Polymerase chain reaction (PCR) amplification of a portion of the cytochrome b gene was performed using the nested PCR

Table I. Samples screened in this study, including the number of positive samples per species.

Species	Number sampled	Positive samples
Ameiva ameiva	6	1
Ameivula ocellífera	4	–
Colobosauroides cearensis	21	–
Enyalius bibronii	20	–
Hemdactylus brasiliana	1	–
Hemidactylus agrius	2	–
Hemidactylus mabouia	20	1
Hemidactylus sp.	1	–
Lygodactylus klugei	11	–
Brasiliscincus aff. heathi	3	–
Copeoglossum nigropunctatum	17	–
Polychrus acutirostris	1	–
Micrablepharus maximiliani	18	–
Phyllopezus pollicaris	20	–
Salvator merianae	2	–
Stenolepsis ridley	22	–
Tropidurus hispidus	18	2
Tropidurus jaguaribanus	1	–
Tropidurus semitaeniatus	18	–

approach outlined in Pacheco et al. (2011). Negative and positive controls were run with each reaction, and products were sequenced by a commercial company (Genewiz, Leipzig, Germany). Electropherograms were checked by eye and compared against published sequences in GenBank using the basic local alignment search tool (BLAST, Altschul et al., 1997). All new sequences were submitted to GenBank (MK033603 to MK033606). New sequences were aligned against published data from GenBank using the ClustalW algorithm implemented in Geneious 4.8.5 (Biomatters Ltd, Aukland, New Zealand). No insertions or deletions were needed in the cytochrome b alignment, which had a total length of 998 base pairs. Since estimates of relationships based on multiple genes indicate that *Plasmodium* from reptiles and birds form a clade (e.g., Borner et al., 2016), representative sequences were included from across these taxa. Following Borner et al. (2016), sequences of *Haemoproteus* species were designated as outgroups.

Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference (BI). In both cases, we implemented the models of sequence evolution selected by PartitionFinder 1.1.1 (Lanfear et al., 2012). ML analyses were performed with RAxML v 7.4.2 (Stamatakis, 2006) with 10 random addition replicates, and node support was estimated with 1,000 nonparametric bootstrap replicates. Bayesian inference was implemented using MrBayes v3.2 (Huelsenbeck and Ronquist, 2001) and run for 1 million generations. After 25% burn-in, remaining trees were combined in a 50% majority-rule consensus.

Of the 194 individuals tested, 4 gave positive PCR products, and the sequence of these amplicons most closely matched those from other lizard-infecting *Plasmodium* species when compared to data from GenBank using BLAST. These came from 3 different host species; 1 individual of *Ameiva ameiva*, 2 individuals of *Tropidurus hispidus*, and 1 individual of the non-native gecko *Hemidactylus mabouia*. All were from Ubajara, Ceará State in Northeast Brazil. In the estimate of phylogenetic relationships (Fig. 1), these new haplotypes fall into 2 different groups. The parasite from *A. ameiva* is most closely related to other forms from *A. ameiva* from Brazil (Perkins and Schall, 2002) and *P. carmelinoi* from *A. ameiva* from Colombia (Matta et al., 2018). Although these are all from the same host species, they show notable genetic distances with this gene (3.4% dissimilarity). The parasites from *T. hispidus* and *H. mabouia* are much more genetically similar to each other (99.8% similarity) and are related to the lineage from *A. ameiva* and to *Plasmodium fairchildi* and *Plasmodium azurophilum*, isolated from anole lizards from the Caribbean (Perkins and Schall, 2002; Falk et al., 2015).

Molecular screening of *Plasmodium* has revolutionized our understanding of the diversity and distribution of these and related parasites in birds. MalAvi, a public database of cytochrome b sequences of *Plasmodium* and related haemosporidian in birds (Bensch et al., 2009) as of March 2018, included 8,266 records from 1,489 host species. By comparison, there are molecular data from just a dozen or so reptiles (e.g., Perkins and Schall, 2002; Falk and Perkins, 2015; Matta et al., 2018), despite the higher number of described *Plasmodium* species from this host group (Telford 2009). Clearly, fewer scientists study blood parasites in reptiles relative to birds, and this explains much of the differences in molecular data availability between the groups. Another partial explanation could be a generally low prevalence of these parasites in reptiles. Sabagh et al. (2015) for example reported very low prevalences of hemoparasites from a lizard assemblage in Southeast Brazil, with no *Plasmodium* detected using microscopy in 128 specimens including *Tropidurus*, *Ameiva* and *Hemidactylus*, species of which were all infected in this study. Other studies in Brazil using microscopy have also reported low prevalence for both *Plasmodium tropiduri* from *Tropidurus* hosts and *P. carmelinoi* from *Ameiva* (Rocha-e-Silva and Rodrigues, 1974; and Lainson et al., 2010, respectively). Our positive records from these hosts seem to corroborate a pattern of low prevalence, at least within the sampled populations from Brazil.

The highly diverse haplotypes recovered from *Ameiva ameiva* indicate that these are from distinct species. Multiple forms have been described from this host, including *P. carmelinoi* (Lainson, Franco and Da Matta 2010), *Plasmodium pifanoi* (Scorza and Dagert 1956), *Plasmodium cnemidophori* (Carini 1941), *Plasmodium attenuatum* (Telford 1973), *Plasmodium telfordi* (Lainson, Landau and Shaw 1971), and *Plasmodium diminutivum* (Telford 1973). The finding of 2 additional genetic lineages related but distinct from *P. carmelinoi* indicates that a single lizard species, *Ameiva ameiva*, already thought to be "host to a surprising number of haematozoa" (Lainson et al., 2010), may host even greater diversity.

Regarding the lizard *T. hispidus*, to the best of our knowledge only *P. tropiduri* has been described from this host. It would seem likely that this corresponds to the 2 similar (99.8%) haplotypes recovered in this study. However, the finding of another almost identical haplotype (99.9%) in *H. mabouia* is unexpected. *Hemidactylus mabouia* is an African species and was first reported in Brazil in 1945; since then it has spread across much of the country (Rocha et al., 2011). To the best of our knowledge, no *Plasmodium* species have been described from *H. mabouia* while hemosporidians identified from other geckos using molecular data

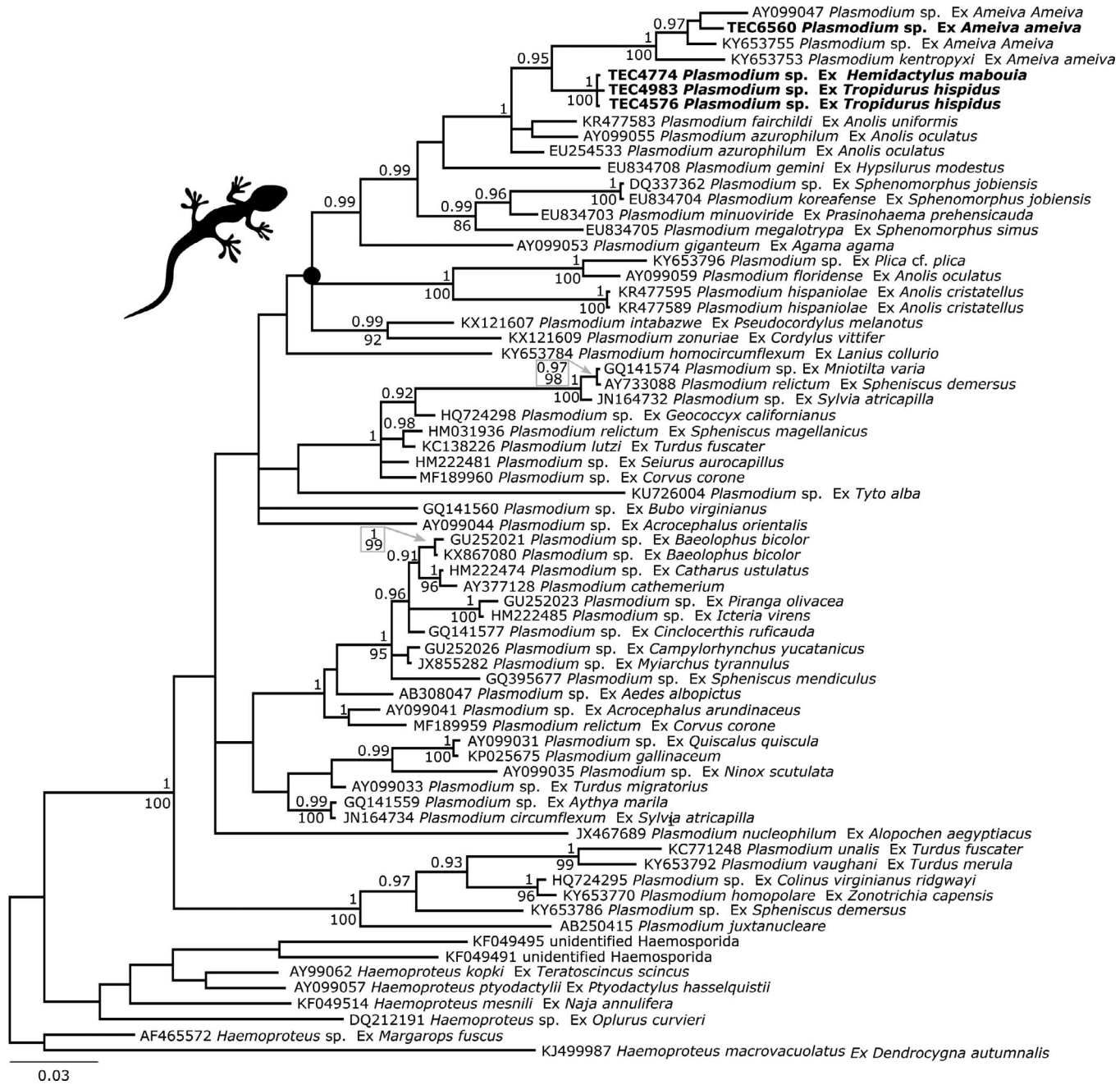

Figure 1. Estimate of relationships of *Plasmodium* species derived from partial cytochrome b gene sequences using a Bayesian approach. Values above nodes correspond to posterior probabilities and those below nodes correspond to bootstrap values from a maximum likelihood approach. New sequences generated from this study are shown in bold.

belong to the unrelated *Haemoproteus* (Fig. 1). It could be that this is an example of the same parasite being found in both *T. hispidus* and *H. mabouia*, but the direction of host-switching is not clear. If the introduced *H. mabouia* also brought new parasites to South America, this would be extremely concerning from a conservation viewpoint. However, without blood smears being available for verification, these results must be considered cautiously, and the possibility of this being a "dead end" infection, where development cannot be completed in a particular host, needs to be evaluated. Still, it does indicate that while a single lizard like *A. ameiva* may host many parasite species, these are not necessarily very host-specific. Indeed, it may be this lack of host specificity, combined with the evidence for undescribed diversity, which has so hampered understanding of hematozoa in reptiles.

Regarding the overall estimate of relationships, since the evidence is from a partial single gene and many nodes are poorly supported, this must be interpreted cautiously. Our estimate of

relationships is quite different from the estimate of relationships based on the same gene of Matta et al. (2018), in which *Plasmodium* from reptiles formed 3 unrelated lineages. Differences in outgroup and ingroup sampling may account for these discrepancies and indicate that as more information becomes available from additional species, and in particular from reptile hosts, current notions of relationships between major lineages within *Plasmodium* may well change.

To conclude, *Plasmodium* species were identified in 3 reptile host species in Brazil and placed in a phylogenetic framework. Recovery of similar haplotypes in the native *T. hispidus* and exotic *H. mabouia* indicate potential host-switching. Prevalence in the screened species, at least in Brazil, seems to be low. The value of molecular screening approaches is again highlighted as well as the need for further studies to identify parasite diversity in this hyperdiverse region.

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