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MOLECULAR SURVEY OF *HEPATOZOON* SPECIES IN LIZARDS FROM NORTH AFRICA

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ABSTRACT: The prevalence of *Hepatozoon* parasites in 460 lizards from North Africa was studied by amplification and sequencing of the 18S rRNA gene. The phylogenetic analysis of the 18S rRNA gene provides new insights into the phylogeny of these parasites with multiple genetically distinct lineages recovered. Parasite prevalence differed significantly between lacertid lizards and geckos. Our results show that there is limited host specificity and no clear relation to the geographical distribution of *Hepatozoon* parasites.

The Apicomplexa, a group of unicellular parasites, is the poorest-studied group of all animals in terms of biodiversity, with only about 0.1% of species described relative to the total number of species estimated (Morrison, 2009). Within Apicomplexa, there is a bias towards a few genera, such as *Plasmodium*, *Babesia*, and *Toxoplasma*, due to their great medical importance and public health consequences. However, several of the most abundant apicomplexan parasites in amphibians, reptiles, and mammals, such as *Hepatozoon*, *Haemogregarina*, *Karyolysus*, or *Hemolivia*, are part of the hemogregarine group (Apicomplexa, Adeleorina) (Smith, 1996; Smith and Desser, 1997; Telford, 2009).

Species of *Hepatozoon* are apicomplexan intracellular parasites included in the Hepatozoidae of hemogregarines and are widely distributed in reptiles, mammals, and amphibians (Telford, 2009). *Hepatozoon* is a very diverse genus with more than 300 species currently assigned to it, based largely on morphological characters, host-specificity, and life cycle patterns (Smith, 1996). Although it is considered as a single genus, due to significant characteristics and diverse life histories of its species, a phylogenetic analysis on morphological and developmental characters by Smith and Desser (1997) suggested it should be partitioned into 2 genera. Moreover, reviews of the current hemogregarine taxonomy using a more integrative approach have revealed that many species seem to be wrongfully classified. The result is many instances of inconsistent phylogenies (Mathew et al., 2000) which have led, for instance, to some species from the other genera, e.g., *Haemogregarina*, being relocated to the genus *Hepatozoon* (see Smith, 1996).

Although fitness-effects are known to be significant in many mammals (Macintire et al., 1997; Baneth et al., 2003; Marchetti et al., 2009), the effects and prevalence in reptiles is poorly known. The few existing studies report very different effects on the hosts ranging from anemia and immunosuppression (Telford, 1984) to no apparent effects (Caudell et al., 2002; for a revision on the effect of several *Hepatozoon* spp. on their hosts, see Telford, 2009). It has been hypothesized that hemogregarines are generally well-adapted parasites that cause little or no pathogenic change in their natural hosts but can cause clinically significant inflammatory disease in unnatural hosts (Wozniak et al., 1994, 1996).

The aim of the present study was to assess *Hepatozoon* spp. prevalence in various reptile groups across the Maghreb, the region of North Africa that includes Morocco, Algeria, and Tunisia, thereby providing new molecular data on reptilian *Hepatozoon* species. Little is still known about hemogregarines of reptiles from North Africa, and there are only a few records from some reptile species, e.g., *Ptyodactylus*, *Tarentola*, and *Hemidactylus* in Saoud et al. (1995), *Psammophis* and *Naja* in Saoud et al. (1996), *Varanus griseus* in Ramdan et al. (1996), and *Ptyodactylus* in Hussein (2006) (all from Egypt), identified using traditional blood smear preparations. However, recent studies have reported a high prevalence of hemogregarines in several lizard species of the Iberian Peninsula such as *Podarcis* (Amo et al., 2004; Roca and Galdón, 2010), *Iberolacerta monticola* (Amo, Lopez et al., 2005), or *Timon lepidus* (Amo, Fargallo et al., 2005). Thus, it is likely that these genera will have at least some infected individuals in the related species existing in North Africa, although prevalence in other groups remains essentially unknown.

Traditionally, diagnostic and species descriptions of *Hepatozoon* spp. were accomplished through the identification of gametocyte morphology in the vertebrate intermediate host and the sporogonic stages in the invertebrate definitive host (Telford et al., 2004; Sloboda et al., 2007). Thus, taxonomic assignment of new species is given according to the host(s) and tissue(s) the parasites inhabit, which makes nomenclature more utilitarian than phylogenetic (Morrison, 2009; but see Smith and Desser [1997] for a phylogenetic approach based on morphological and developmental characters). In fact, the occurrence of gametocytes in a new host has often been used to justify the description of a new species (Ball, 1967; Sloboda et al., 2007). However, given that parasites may infect a wide range of host species, it has been suggested that molecular tools which can be used to assess the phylogenetic relationships between parasites should also be included for diagnostic and taxonomic purposes (Telford et al., 2004).

Various studies indicated that detection of parasites from blood or tissue samples, using PCR protocols followed by DNA sequencing, is at least as effective as examination of blood smears, if not more so, as it can detect low levels of parasitemia and distinguish between species or strains through the detection of polymorphisms (e.g., Ujvari et al., 2004; Criado-Fornelio et al., 2007; Merino et al., 2009; Gabrielli et al., 2010; Harris et al., 2011). At the same time, after this methodology is optimized, it becomes an easier and faster method with which to assess prevalence on larger numbers of individuals.

Here, 460 reptile samples from North Africa were assessed using *Hepatozoon*-specific primers that amplify a region of 18S rRNA. By comparing various families and genera of reptiles, parasite prevalence could be compared at different host taxonomic levels. A phylogenetic analysis was performed to clarify how *Hepatozoon* spp. from this region were related to known *Hepatozoon* spp. from other reptiles and vertebrate hosts.

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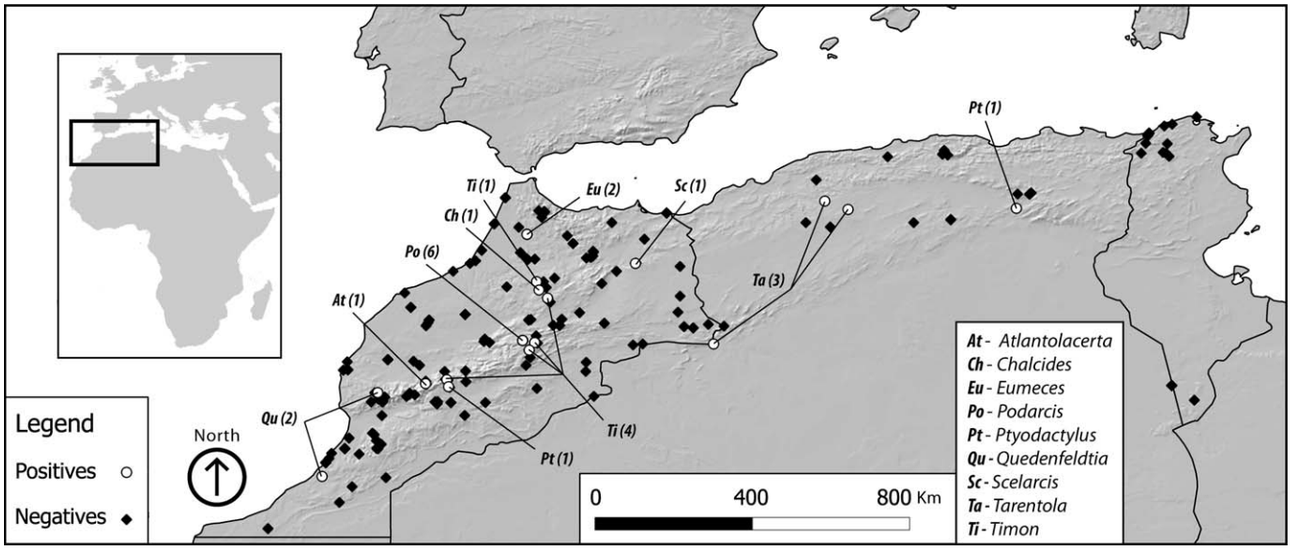

FIGURE 1. Map of North Africa containing the location of the analyzed samples in this study. Dark squares indicate samples negative for *Hepatozoon* whereas white dots indicate positive samples. Species designation and the number of positive individuals (in brackets) are given for each region. The map was done using the Quantum GIS (ver. 1.5.0., 'Tethys') software.

MATERIALS AND METHODS

Sample collection

Tissue samples from lizard specimens collected between 2003 and 2009 across the Maghreb (see Fig. 1), and preserved for molecular analysis (tail tips containing blood stored in 96% ethanol), were used to detect the presence of *Hepatozoon* parasites. In all cases, lizards were collected, identified, digitally photographed, and their location registered using a GPS device. Afterwards, they were released at the capture place. A total of 460 tissue samples were collected: 19 from Tunisia, 21 from Algeria, and 420 from Morocco, comprising a total of 9 genera from 3 lizard families (see Table I). More details regarding these locations are published in Harris et al. (2008, 2010).

DNA extraction, amplification, and sequencing

DNA was extracted from tissue using DNeasy Blood & Tissue kit (Qiagen, Washington, D.C.) following the manufacturer's instructions or by using standard high salt methods (Sambrook et al., 1989). Detection of *Hepatozoon* parasites was initially made using PCR reactions, with the hemogregarine-specific primers HEMO1 and HEMO2 targeting part of the 18S rRNA region following Perkins and Keller (2001). Samples were then used in a further PCR reaction using the primers HepF300 and HepR900, targeting another part of the 18S rRNA region following Ujvari et al. (2004). PCR conditions for both fragments are detailed in Harris et al. (2010). Negative and positive controls were run with each reaction. The positive PCR products obtained were purified and sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea). All fragments were sequenced in both directions.

TABLE I. Family and genus of *Hepatozoon* hosts included in this study. For each genus, the total number of individuals analyzed and the number of infected samples are given, as tested through PCR amplification (PCR). Observed prevalence is calculated using the total number of lizard hosts analyzed and the total number infected.

Family	Genus	Total analyzed	Total infected	Observed prevalence (%)
Scincidae	*Chalcides*	68	1	1.5
	Eumeces	15	2	13.3
Lacertidae	*Atlantolacerta*	50	1	2.0
	Timon	35	5	14.3
	Podarcis	34	6	17.6
	Scelarcis	23	1	4.3
Gekkonidae	*Ptyodactylus*	26	2	7.7
	Quedenfeldtia	56	2	3.6
	Tarentola	153	3	2.0
		460	23	5.0%

Phylogenetic analysis

Consensus sequences for each individual were created by combining the sequences of the 2 partially overlapping 18S rRNA regions, thereby obtaining a total of 23 parasite sequences and 12 unique haplotypes. Sequences were blasted in GenBank and all of them matched known *Hepatozoon* spp. sequences. These were aligned with 22 *Hepatozoon* sequences retrieved from GenBank (see GenBank accession numbers and more details in Harris et al., 2010). This included all available sequences that were as long as those generated for this study, except for *H. canis* and *H. felis*, for which a representative subset was included. Sequences were aligned using ClustalW software implemented in the program BioEdit (Hall, 1999). The final dataset contained 45 *Hepatozoon* sequences approximately 1,400 bp in length.

Three different phylogenetic analyses (maximum likelihood [ML], maximum parsimony [MP], and Bayesian inference [BI]) were conducted. ML analysis with random sequence addition (100 replicate heuristic searches) was used to assess their evolutionary relationships. Support for nodes was estimated using the bootstrap technique (Felsenstein, 1985) with 1,000 replicates. The AIC criteria carried out in Modeltest 3.06 (Posada and Crandall, 1998) was used to choose the model of evolution employed. MP analysis was performed in PAUP v. 4.0b10 (Swofford, 2002) with 1,000 replicate heuristic searches and support estimated using the bootstrap technique. BI analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 5×10^6 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 Morrison

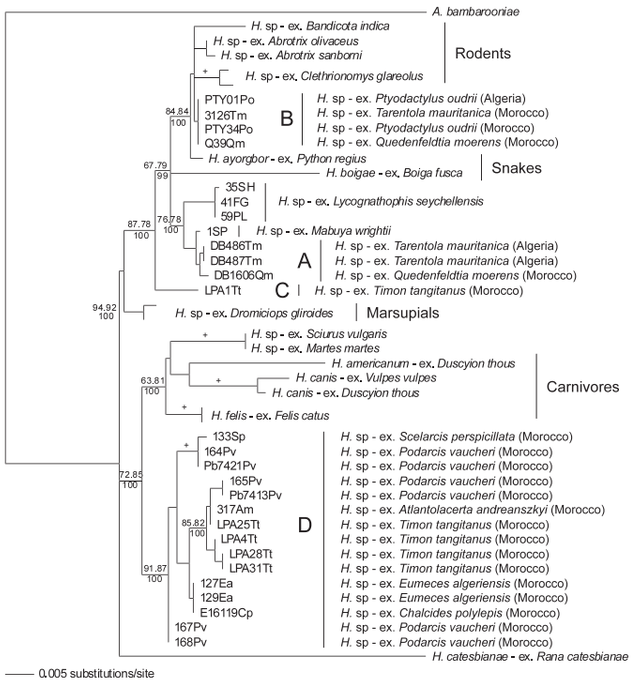

FIGURE 2. Tree derived from a maximum likelihood (ML) analysis using the GTR+I+γ model. The consensus tree of 3,432 maximum parsimony (MP) trees (407 steps) differed only in being less well-resolved. Bootstrap values for MP and ML are given above relevant nodes, respectively, and Bayesian posterior probability appears below them. When all values were 100%, this is indicated with a "+" symbol.

(2009), *Adelina bambarooniae* was used as an outgroup for rooting the phylogenetic tree.

RESULTS

Of the 460 individuals analyzed, 23 were found to be infected with *Hepatozoon* parasites, resulting in an overall prevalence of 5%. Prevalence was not uniformly distributed among the different genera analyzed (Table I). It was lower among *Chalcides* (Scincidae), *Tarentola* (Gekkonidae), and *Atlantolacerta* (Lacertidae) with 1.5%, 2%, and 2%, respectively, and was higher among *Timon* and *Podarcis* (Lacertidae) with 14.3% and 17.6%, respectively. Assessing the 2 families with greater sampling (Lacertidae and Gekkonidae), the number of positives found (13 and 7, respectively) were significantly higher for lacertids and lower for geckos (χ^2 test, $P < 0.05$) than the ones that would be expected if prevalence was uniform (7.5 and 12.5, respectively).

All phylogenetic methodologies produced the same estimate of relationships among the *Hepatozoon* sequences of the 18S rRNA gene (Fig. 2). Our results show the existence of 4 main lineages in the Maghreb region; 2 are composed of sequences from parasites of geckos (species of *Ptyodactylus*, *Tarentola*, and *Quedenfeldtia* – lineages A and B), 1 of a single *Hepatozoon* isolate of a lacertid (*Timon* sp. – lineage C) and the other of lacertids (*Atlantolacerta*, *Timon*, *Podarcis*, and *Scelarcis* – lineage D) and skinks (*Eumeces* and *Chalcides* species) (see Fig. 2).

The first lineage found in geckos (lineage A in Fig. 2, from *Tarentola* and *Quedenfeldtia* with 2 isolates from Algeria and 1 from Morocco, respectively) is closely related to *Hepatozoon* spp. found in skinks (*Mabuya wrightii*) and snakes (*Lycognathophis seychellensis*) from the Seychelles Islands. A second lineage found in geckos (lineage B in Fig. 2, from *Ptyodactylus* sp. from Algeria and Morocco and species of *Tarentola* and *Quedenfeldtia* from Morocco) is, however, more closely related to *Hepatozoon* found in members of the Rodentia from Chile, Spain, and Thailand and to *Hepatozoon ayorborg* reported from a royal python, *Python regius*. The lineage with a single *Hepatozoon* sequence from a lacertid host from Morocco (lineage C in Fig. 2, from *Timon* sp.) is surprisingly not related to other lacertids and is sister taxa to the group comprising *Hepatozoon* spp. from rodents and most reptiles, including the 2 previously described lineages from geckos. Finally, the fourth identified lineage (lineage D in Fig. 2, with all the other 15 isolates [8 haplotypes] from lacertids and skinks from Morocco) forms a distinct clade from the other reptiles, sister taxa to a clade that includes *Hepatozoon* spp. reported from carnivores (including *H. felis*, *H. canis*, and *H. americanum*) and undetermined *Hepatozoon* sp. from a squirrel and pine marten. There can be highly divergent lineages of *Hepatozoon* spp. infecting the same host species (e.g., species in *Quedenfeldtia*, *Podarcis*, and *Timon*). On the other hand, *Hepatozoon* spp. of *Chalcides* spp. share the same haplotype with species of *Hepatozoon* in *Eumeces* spp., as do species from *Atlantolacerta* and *Timon* (Fig. 2).

DISCUSSION

Hepatozoon spp. prevalence has been assessed for the first time among different lizard families across the Maghreb region of North Africa. Our results show that the occurrence of *Hepatozoon* species varies significantly among lizard families, with lacertids showing higher prevalence values than do geckos. In fact, species of *Podarcis* and *Timon* show the highest prevalence values, i.e., 17.6% and 14.3%, respectively. Nonetheless, species sampling was not uniform across regions, with a high number of infected individuals from 1 species being collected from a single region, e.g., *Podarcis* sp., whereas individuals from other species that are widespread had few individuals sampled from each location, e.g., species of *Ptyodactylus* and *Tarentola*. This is clearly reflected in the number of positives from each location (see Fig. 1) and, thus, further research using more uniform sampling of groups from each region is needed for a comparison between parasite prevalence and location.

Furthermore, prevalence in the present study is lower than that found in a recent morphological survey of hemogregarines in *Podarcis bocagei* and *Podarcis carbonelli* from the Iberian Peninsula by Roca and Galdón (2010) (74.7% and 69.7%, respectively, based on blood smear surveys). Despite the different methodologies used in both studies, the relatively high prevalence of *Hepatozoon* spp. in *Podarcis* and *Timon* spp. suggest these can be promising populations for further studies of *Hepatozoon* spp. infections and their impacts to hosts.

Other studies have also found very high prevalence values, such as Telford et al. (2004), which found 66% of prevalence in 104 snakes in North Florida and Ujvari et al. (2004), which found 100% prevalence using molecular techniques in 100 water pythons from tropical Australia. Prevalence among snakes seems to be higher than among lizards, which may indicate that *Hepatozoon* spp. infections are age-related considering that snakes, in general, live much longer than do lizards. However, *Hepatozoon* spp. prevalence varies depending on the testing methodology used

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(molecular methods versus blood smear scanning), with different studies reporting different degrees of congruence between both techniques (Ujvari et al., 2004; Criado-Fornelio et al., 2007, 2009; Valkiunas et al., 2008; Harris et al., 2010). In the case of the molecular assessment, factors include the DNA extraction methodology and the type of primers and PCR protocols used, which can have different sensitivities (Valkiunas et al., 2008). Moreover, independent of the methodology applied, the host-related variables, e.g., age or size (see Brown et al., 2006 and Salkeld and Schwarzkopf, 2005) or the time of year of sample collection (Salkeld and Schwarzkopf, 2005; Santos et al., 2005; and Huyghe et al., 2010) are other factors that must be considered when comparing prevalence levels from different species. Most of the samples used in this study were collected in April–May, which does not allow a comparison of seasonal changes; thus, further research should include different seasonal collections.

Our findings show that *Hepatozoon* lineages are very complex, with great variation within and between hosts. Multiple, closely related haplotypes were found in species of *Chalcides*, *Eumeces*, *Atlantolacerta*, *Timon*, and *Podarcis*, constituting a completely new genetic *Hepatozoon* lineage. There is also limited host specificity, with similar *Hepatozoon* spp. isolates infecting different genera of lizards. These results seem to indicate that some *Hepatozoon* spp. infections are not host-specific and that the parasite has the ability to switch easily between different host species; therefore, identification of new *Hepatozoon* species based on detection of gamonts in new hosts should be done with caution. In fact, successful experimental transmissions in the laboratory have demonstrated that *Hepatozoon* spp. host-specificity is low. For instance, mosquitoes that fed on snakes infected with *Hepatozoon* spp. were given to lizards that developed short-term parasitemia, as demonstrated by Booden et al. (1970). Ujvari et al. (2004) found that similar *Hepatozoon* nucleotide sequences (0–0.029 pairwise differences) were present in different host species and squamate families, and Telford et al. (2008) described a cross-familial transfer of *Hepatozoon* spp. among natural populations of snakes. Nonetheless, other studies have demonstrated that some other species of *Hepatozoon* are narrowly host-specific; for example, Telford et al. (2001) reported 5 species of *Hepatozoon*, each from a single host species. Strong co-evolutionary relationships may be associated with some degree of host-specificity of parasites regarding the definitive hosts (Carreño et al., 1997). Therefore, further studies should elucidate relationships among lizard hosts, and among their *Hepatozoon* spp. vectors of lizards which include ticks, mites, and mosquitoes.

The fact that some species of *Hepatozoon* seem to have limited host-specificity has led parasitologists to hypothesize that the *Hepatozoon* spp. host spectrum is limited to the host ecology rather than to host phylogenetic relationships (Sloboda et al., 2007; Vilcins et al., 2009). In the present study, this is not supported because it is not clear how parasites from different geographical locations are grouped together, in particular how some *Hepatozoon* isolates of geckos from North Africa are more related to isolates found in reptiles from the Seychelles than to other lizards from North Africa. Thus, the new data suggest that *Hepatozoon* isolates from the Seychelles are not monophyletic, as was previously proposed by Harris et al. (2010). Moreover, given that only molecular data were used in this study and that for correct species recognition microscopic examination should also be used, one cannot discard the possibility of lineages C and D belonging to other hemogregarines, such as species of *Karyolysus*, *Haemogregarina*, or *Hemolivia*, which are also abundant in reptiles. However, given that these lineages fall within a clade consisting of known *Hepatozoon* isolates, there is no reason to assume they are not species of *Hepatozoon*.

Although *Hepatozoon* spp. infections show limited host-specificity and no clear relation with host ecology as previously hypothesized, the relationships of *Hepatozoon* isolates in the Maghreb region remains largely unresolved. The lack of parasite variability among species of *Chalcides* and *Eumeces* should be further investigated to determine if these hosts are reservoirs for the same *Hepatozoon* species. Prevalence assessed with molecular methods varies significantly among different lizard families, which may indicate that *Hepatozoon* spp. infection or detection could be connected to host-related variables such as age or size or to more general factors such as parasite seasonal variation; however, more investigation is needed here. Finally, further research should also include the design of primers to target faster-evolving genes; these would be used in parallel with the already established 18S rRNA studies to better resolve relationships within the already identified *Hepatozoon* lineages.

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