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MOLECULAR SURVEY OF APICOMPLEXA IN *PODARCIS* WALL LIZARDS DETECTS *HEPATOZOON*, *SARCOCYSTIS*, AND *EIMERIA* SPECIES

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ABSTRACT: The occurrence of apicomplexan parasites in *Podarcis* sp. wall lizards from the Iberian Peninsula and Balearic islands was studied by amplification and sequencing of the 18S rRNA gene. Species from 3 genera, *Hepatozoon*, *Sarcocystis*, and *Eimeria*, were found. The phylogenetic analysis of the 18S rRNA gene provides unexpected insights into the evolutionary history of these parasites. All *Hepatozoon* spp. specimens were recovered as part of a clade already identified in lizards from North Africa. The *Sarcocystis* species, detected in *Podarcis lilfordi* from Cabrera Island in the Balearic Islands, appears related to *Sarcocystis gallotiae*, known only from endemic *Gallotia* sp. lizards from the Canary Islands. Based on the lack of snake predators on this island, this parasite presumably presents an atypical transmission cycle that uses the same host species as both intermediate and final host through cannibalism, like *S. gallotiae*. *Eimeria* sp. is reported for the first time from *Podarcis* spp. lizards. This study shows the power of detecting multiple different apicomplexan parasites through screening of tail tissue samples and blood drops that are often collected in reptiles for other purposes.

The Apicomplexa includes a diverse group of unicellular parasites that is possibly the poorest-studied group of all animals in terms of biodiversity (Morrison, 2009). Yet, according to the European Cooperation in Science and Technology (COST), apicomplexan protozoa caused more human deaths than any other group of infectious agents and are also the most significant parasites of livestock. Although molecular techniques have now become established as standard tools for monitoring parasite populations (Beck et al., 2009), this is heavily biased to certain groups within Apicomplexa, such as *Plasmodium* spp., and is also directed primarily toward humans or commercially important animal groups. However, parasites also have dual interests for conservation biologists. On the one hand, parasite-driven declines in wildlife are becoming common (Pedersen et al., 2007), whereas on the other hand, parasites themselves are also a major component of biodiversity (Poulin and Morand, 2004). Because of their obligate relationship with a host, parasites are especially at risk through coextinction, where the loss of 1 species leads to the loss of another, so that an extinction cascade can occur. Models suggest that coextinction may be very common (Dunn, 2009), but lack of knowledge hinders assessments.

Reptiles are hosts to a wide variety of apicomplexan parasites, including families with human medical importance such as Sarcocystidae (which includes *Toxoplasma*), Eimeriidae, e.g., *Cryptosporidium parvum*, and Haemosporidae, e.g., *Plasmodium* spp. The most common and widely distributed apicomplexans found in reptiles are species of *Hepatozoon* (Telford, 2009). Traditionally, assessment of gametocyte morphology in the vertebrate host and sporogonic stages in the invertebrate host was used for diagnosis and species description. This situation is complicated, however, when only information from the vertebrate host is available, which is often the case. Fortunately, for *Hepatozoon* species, universal primers are available that can amplify part of the 18S rRNA gene. These have been used to assess prevalence, estimate phylogenies, and confirm distinctiveness in a variety of vertebrate hosts, including mammals (Merino et al., 2009) and reptiles (Harris et al., 2011; Maia et al., 2011). However, although for mammals there is a wide knowledge regarding other apicomplexan groups, much less information is available for reptiles. *Eimeria* species, for example, cause coccidiosis and are the most important protozoan pathogens of poultry (Beck et al., 2009). Not surprisingly, there is considerable molecular data available for both domestic (Barta et al., 1997; Miska et al., 2010) and wild (Honma et al., 2007) birds, but only a single 18S rRNA sequence is available from a species that infects reptiles, i.e., *Eimeria arnyi* (Upton and Oppert, 1991). Similarly, Sarcosporidia are prevalent around the world, affecting amniotic vertebrates, including humans. Species of *Sarcocystis* have a diheteroxenous cycle and, for most described species, mammals and reptiles are both intermediate and final hosts. However, some insular species such as *Sarcocystis gallotiae* or *Sarcocystis dugesii* that infect lizards endemic to the Canary and Madeira Islands, respectively, are atypical, because the same lizard species is both intermediate and final host, which occurs via cannibalism (Matuschka and Bannert, 1987; Matuschka, 1988). Various other species have a snake–lizard life cycle (reviewed in Matuschka, 1987). Again, however, despite a wide variety of species from mammals and birds having been sequenced for the 18S rRNA gene, often to confirm the distinctiveness of new species (Kutkiene et al., 2010; Xiang et al., 2010; Kutkiene et al., 2011), only a couple of studies have included such data from reptile species (Šlapeta et al., 2001, 2003).

The aim of the present study was to screen populations of *Podarcis* spp. wall lizards (Lacertidae) from the Iberian Peninsula and the Balearic Islands for apicomplexan parasites with the use of 18S rRNA primers. Several *Podarcis* spp. from the Iberian Peninsula have been shown to have high levels of infection with hemogregarines with the use of traditional blood smears (Álvarez-Calvo, 1975; Amo et al., 2005; Roca and Galdón, 2010), and 18S rRNA primers are known to amplify *Hepatozoon* spp. from lacertids in North Africa, including *Podarcis vaucheri* (Maia et al., 2011). At the same time, these lizards were assessed with the use of autotomized tail samples that are typically used in phylogeographic studies of the host (Pinho et al., 2011). *Sarcocystis* spp. have been reported to infect several species of *Podarcis,* including *Podarcis lilfordi* endemic to the eastern Balearic Islands (Mayo et al., 1988) and classified as endangered on the IUCN red list (Pérez-Mellado and Martínez-Solano, 2009), among others (see a complete review in Odening, 1998). Because sarcocysts are typically observed in tail muscle in lizards (Abdel-Ghaffar et al., 2009), there is also a reasonable possibility that these will be detected by molecular methods. Additionally, a possible amplification of an apparent

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TABLE I. Summary of the 44 samples analyzed in this study, including positives for parasites and their geographical locations. Coordinates are in the WSG84 format.

	Host species			
	Podarcis hispanica	*Podarcis hispanica*	*Podarcis lilfordi*	
	Portugal (40.83°, −5.52°)	Spain (38.02°, −7.86°)	Cabrera (39.15°, 2.93°)	Total
Hepatozoon	0	17* (89.5%)	7 (70%)	24
Sarcocystis	0	0	8 (80%)	8
Eimeria	1 (6.7%)	0	0	1
Total	15	19	10	

* Two samples were positive with only the HEP primers and therefore were not included in the analyses.

member of the Eimeriidae was reported with these primers within reptiles from the Seychelles (Harris et al., 2011), and thus the possibility exists of detecting these, or even other, apicomplexan parasites. In the present study, we use molecular methods to assess the existence of different apicomplexan species using 18S rRNA primers. Positive samples were sequenced and included in a phylogenetic assessment with previously published sequences to help determine the evolutionary history and patterns of genetic diversity within these parasite species.

MATERIALS AND METHODS

Sample collection

Samples from lizard specimens were collected from 2 localities in the Iberian Peninsula (April 2008 in Beja, Portugal, and June 2009 in Alba de Tormes, Spain) and from Cabrera Island, Balearic Islands (September 2010). Tail tips from the lizards were preserved in 96% ethanol for molecular analysis and blood drops were placed in Whatman paper. When lizards autotomize their tails, very little blood is typically lost and, in most cases, there was not enough to make blood slides. After samples were collected, the lizards were released at the site of capture. A total of 44 tissue samples was collected, i.e., 10 from Cabrera (*P. lilfordi*), and 34 from the 2 localities on the Iberian Peninsula (*Podarcis hispanica*) (see Table I).

DNA extraction, amplification, and sequencing

DNA was extracted from tissue and blood drops with the use of standard High Salt methods (Sambrook et al., 1989). Detection of parasites was made with the use of polymerase chain reactions (PCRs) with the primers HEMO1 and HEMO2 (Perkins and Keller, 2001) targeting part of the 18S rRNA region, and with the primers HepF300 and HepR900 (Ujvari et al., 2004) targeting another partially overlapping part of the 18S rRNA region. Conditions of the PCR are detailed in Harris et al. (2011). Briefly, PCR cycling for the HEMO primers consisted of 94 C–30 sec, 48 C–30 sec, 72 C–1 min (35 cycles), and for the Hep primers annealing temperature was 61 C. Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing service (Macrogen Inc., Seoul, Korea). All sequences were performed in both directions. Sequences were deposited in GenBank under the accession numbers JQ762306 to JQ762311.

Phylogenetic analysis

Because considerable genetic differences between the parasites were detected, and because different parasites were detected with the alternative primer pairs (see results), separate phylogenetic analyses were performed for each of the parasite groups recovered. For each data set new sequences generated in this study were aligned with related sequences retrieved from GenBank. All sequences for which the full length was available were included in the analyses, except for cases in which many closely related sequences are available for some taxa, e.g., *Eimeria reichenowi*. In these cases, single exemplars were arbitrarily used. Sequences were aligned with the use of ClustalW software implemented in the program BioEdit (Hall, 1999). The final 3 data sets contained 47, 21, and 12 taxa that were 798, 609, and 636 base pairs (bp) in length, respectively.

Maximum likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to estimate their evolutionary relationships with the use of the software PAUP (Swofford, 2002). Support for nodes was estimated with the use of the bootstrap technique (Felsenstein, 1985) with 1,000 replicates. The model of evolution employed, in all cases GTR with an estimate of invariant sites and a gamma distribution of site variation, was chosen with the use of the AIC criteria carried out in Modeltest 3.06 (Posada and Crandall, 1998). Bayesian analysis was implemented with the use of Mr. Bayes v.3.2 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 1×10^7 generations, saving 1 tree every 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 as burn-in samples. Remaining trees were combined in a 50% majority consensus tree. Following Maia et al. (2011) and Morrison (2009), *Adelina bambarooniae* was used as an outgroup for rooting the phylogenetic tree for the *Hepatozoon* spp. sequences. As in Šlapeta et al. (2003), species of *Besnoitia* and *Hylokossia* were used to root the estimate of relationships of the *Sarcocystis* spp., whereas, as in Jirku et al. (2009), *Choleoeimera* sp. and *Eimeria tropidura* were used to root the estimate of relationships of the *Eimeria* spp. data set.

RESULTS

Of the 44 individuals analyzed, 24 were found to be infected with *Hepatozoon* sp. parasites, with the use of the HEMO primers. This included infections in *P. hispanica* (0% in Portugal and 89.5% in Spain) and *P. lilfordi* (70% in Cabrera) (Table I). Two individuals negative for the HEMO primers were found to be infected with *Hepatozoon* sp. with the use of the HEP primers. Both phylogenetic methodologies produced the same estimate of relationships among the *Hepatozoon* sp. sequences of the 18S rRNA gene (Fig. 1). All of the *Hepatozoon* sp. lineages identified in *Podarcis* spp. from the Iberian Peninsula and the Balearics formed part of a group composed of 1 of the major clades in North Africa, detected in various lacertids (species of *Timon*, *Podarcis*, and *Scelarcis*) and skinks (species of *Eumeces* and *Chalcides*), and a clade of mammalian *Hepatozoon* sp. isolates (Fig. 1).

Within *P. lilfordi*, several individuals (80%) were found to be infected with *Sarcocystis* sp. with the use of the HEP primers, although no infection was reported in the other 2 localities analyzed (Table I). Some of these (5) were also infected with *Hepatozoon* sp. parasites, but there was no obvious double amplification; i.e., with the HEMO primers only *Hepatozoon* sp. were amplified, and with the HEP only *Sarcocystis* sp. were amplified. The sequences amplified group with the ones available in GenBank for other lacertid species (Fig. 2). Interestingly, the *Sarcocystis* sp. from *P. lilfordi* was most closely related to *S. gallotiae* from *Gallotia* sp.

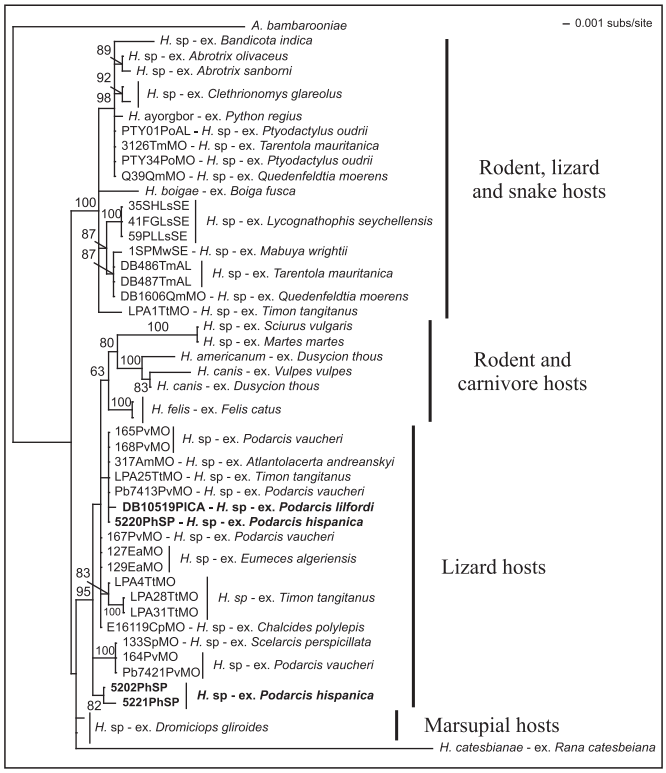

FIGURE 1. Tree derived from a maximum-likelihood (ML) analysis of the *Hepatozoon* sp. sequences. New haplotypes from this study are in bold. For all other codes refer to Maia et al. (2011).

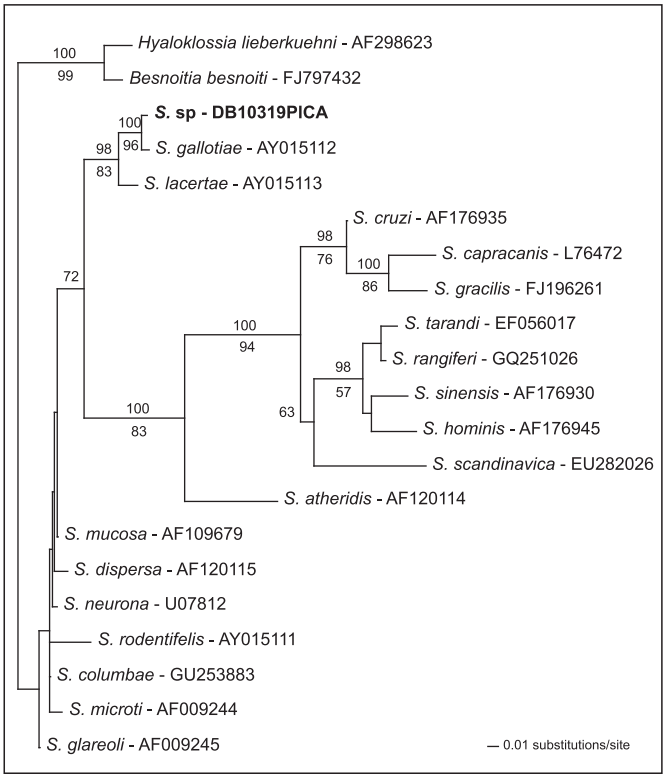

FIGURE 2. Tree derived from a maximum-likelihood (ML) analysis of the *Sarcocystis* sp. sequences. Bootstrap values for ML are given above relevant nodes, and Bayesian posterior probability below them. New haplotypes from this study are in bold.

lizards, rather than *Sarcocystis lacertae*, which infects *Podarcis muralis* (Fig. 2).

Also using the HEP primers, a single individual of *P. hispanica* from Portugal was found to be infected with *Eimeria* sp. (Table I). Although there is limited available comparative data for *Eimeria* spp. from reptiles, the sequence was determined to be most closely related to *E. arnyi* from a snake, and then to *Eimeria ranae* from a frog (Fig. 3). To our knowledge, this is the first report of *Eimeria* sp. infection in *Podarcis* sp. lizards.

Thus, in this study HEP primers amplified *Hepatozoon*, *Sarcocystis*, and *Eimeria* species, although HEMO primers only amplified *Hepatozoon* sp. In dual-infected *P. lilfordi*, HEP primers preferentially amplified *Sarcocystis* sp., whereas HEMO amplified *Hepatozoon* sp.

DISCUSSION

For the first time, *Hepatozoon* sp. from *Podarcis* spp. from the Iberian Peninsula and the Balearic Islands can be placed in a phylogenetic framework. Despite the shorter sequences used in this study (about 800 bp), the overall estimate of phylogeny is essentially the same as that found by Maia et al. (2011), using about 1,400 base pairs, although with lower levels of statistical support. All the newly sequenced parasites form part of a group that includes a variety of lacertids and skinks from North Africa (Maia et al., 2011). The 4 *Hepatozoon* spp. haplotypes recovered in this study do not form a clade, but are instead related to various haplotypes from North Africa and to mammalian isolates. Although this increases the known geographic distribution of this group, and the number of known host species, the inclusion of new data has little influence on the overall phylogeny of *Hepatozoon* spp. This lineage, previously known only from reptiles, appears to be related to a lineage that includes *Hepatozoon canis*, *Hepatozoon americanum*, and *Hepatozoon felis*, which usually use carnivores, including cats and dogs, as their intermediate hosts. The present study also confirms that closely related *Hepatozoon* spp. haplotypes can be found in unrelated hosts, i.e., in this case, species of *Chalcides*, *Eumeces*, *Atlantolacerta*, *Scelarcis*, and *Timon*, and at least 3 *Podarcis* species. Clearly, there is a difference in specificity between the pairs of primers used, with Hep primers amplifying samples that failed to amplify with the partially overlapping HEMO primers. The Hep primers are less specific, also amplifying other apicomplexans. It is, therefore, inappropriate to compare prevalence directly across studies using these different markers.

Mayo et al. (1988) first reported *S. dugesii* from *P. lilfordi* on Cabrera Island. Until then, *S. dugesii* had been only known from the Madeiran endemic lacertid *Teira dugesii* (Matuschka and Mehlhorn, 1984), and later reviews report only a form "similar to *S. dugesii*" in *P. lilfordi* (Odening, 1998). Given that *Sarcocystis* spp. show some signs of coevolution with their snake hosts (Šlapeta et al., 2003), it could be expected that the form in *P. lilfordi* would be more similar to *S. lacertae*, known from *Podarcis muralis*. However, in our estimate of relationships, *Sarcocystis* spp. from *P. lilfordi* are clearly more closely related to *S. gallotiae* from *Gallotia* spp. lizards endemic to the Canary Islands. *Sarcocystis gallotiae* and *S. dugesii* are notable because they use the same lizards as both intermediate and final host, a life cycle described as dihomoxenous (Matuschka, 1988), whereas *Sarcocystis lacertae* has a more typical

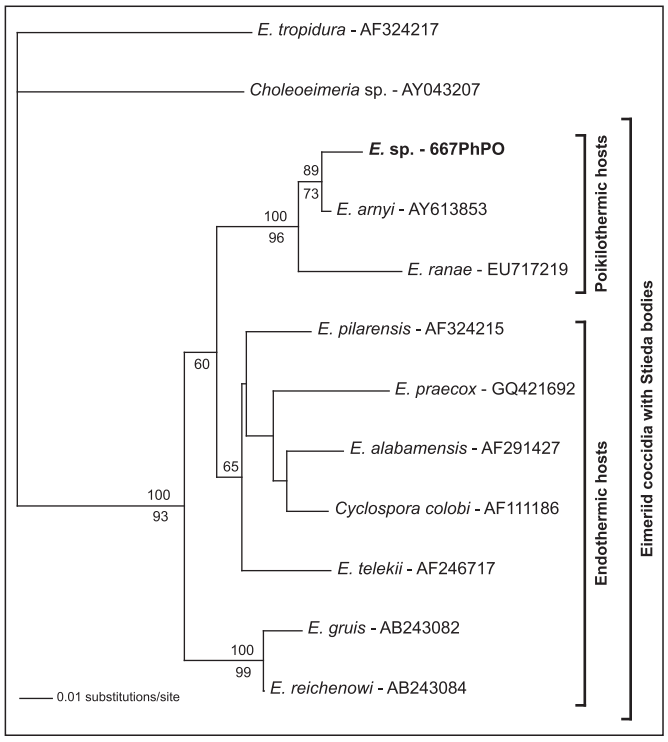

FIGURE 3. Tree derived from a maximum-likelihood (ML) analysis of the *Eimeria* sequences. Bootstrap values for ML are given above relevant nodes, and Bayesian posterior probability below them. New haplotypes from this study are in bold.

snake (*Coronella austriaca*) –lizard (*P. muralis*) life cycle (Volf et al., 1999). Given that there are no snake or mammalian predators on Cabrera Island, the *Sarcocystis* sp. found in *P. lilfordi* are similarly likely to use the lizard as both intermediate and final host. Indeed, cannibalism is in general more common in island lizards where resources are limited (Pafilis et al., 2009). Although these are the only species known to have always the same intermediate and final host, 2 other species, *Sarcocystis rodentifelis* and *Sarcocystis muris*, can occasionally exhibit transmission from an intermediate host to a paratenic host through cannibalism (Šlapeta et al., 2001). In a concatenated analysis of large and small subunit rRNA genes, Šlapeta et al. (2003) found these 2 species to be sister taxa, and related to a lineage of *S. lacertae* and *S. gallotiae*, although in our analysis the phylogenetic position of *S. rodentifelis* is not well supported. The finding of another dihomoxenous form related to *S. gallotiae* raises important evolutionary questions. First, it implies that dihomoxeny may have arisen just once as a facultative strategy that became fixed in island host species that had no alternative predator to fill the niche of final host. This would mean that a reversal to a diheteroxenous life cycle occurred in *S. lacertae*. Alternatively, dihomoxeny could have arisen twice, once in the lineage leading to *S. muris* and *S. rodentifelis*, and again in the lineage leading to *S. gallotiae* and the form found in *P. lilfordi*. Second, the relationship of a parasite lineage found in 2 geographically separated island endemic hosts implies that the lineage is probably old. *Gallotia* spp. separated from other ancestral lacertids when they colonized the Canary Islands perhaps 17–20 MYA (Cox et al., 2010). Similarly, *P. lilfordi* has been separated since the time of the Messinian Salinity Crisis, approximately 5.3 MYA (Brown et al., 2008). To address both these questions relating to the origins of dihomoxeny and its age fully, other closely related (and possibly conspecific) *Sarcocystis* species need to be included in the phylogenetic framework, in particular *S. dugesii* from *T. dugesii* in Madeira and *Sarcocystis podarcicolubris*, another *Sarcocystis* species known from *Podarcis* spp. that has a diheteroxenous life cycle (Matuschka, 1985).

A single specimen of *P. hispanica* from Portugal was shown to be infected with *Eimeria* sp. with the use of the HEP primers. This is again relevant for various reasons. First, it is, to our knowledge, the first report of *Eimeria* sp. from *Podarcis* sp. There is an old record of *Eimeria* sp. in another lizard that occurs in the Iberian Peninsula, *Zootoca vivipara* (formerly *Lacerta vivipara*) from 1935 (Yakimoff and Gousseff, 1935). Additionally, there are a few reports of *Eimeria* sp. in lacertids from North Africa (*Acanthodactylus* sp.; Sakran et al., 1994; Al Yousif et al., 1997), Asia (*Eremias* sp. and *Takydromus* sp. [Davronov, 1985; Telford, 1992, respectively]), and from the Canary Islands (*Gallotia* sp. [Matuschka and Bannert, 1987]). Second, there were previously almost no molecular data regarding *Eimeria* sp. from amphibians and reptiles, with only a couple of sequences previously published (Šlapeta et al., 2001, 2003).

The estimate of relationships presented here (Fig. 3) indicates that the species of *Eimeria* from *Podarcis* spp. is most closely related to *E. arnyi*, the only other analyzed specimen from a reptile host. In our analysis, *Eimeria* spp. from poikilothermic hosts form a monophyletic group, whereas those from endothermic hosts are paraphyletic, because the clade that includes *Eimeria gruis* and *E. reichenowi*, both from cranes, is basal to all ingroup taxa, although these relationships are only weakly supported. This differs from the estimate of relationship of Jirku et al. (2009), who found that the *Eimeria* spp. from endothermic hosts formed a monophyletic group. Jirku et al. (2009) unfortunately did not include any members of the *E. gruis*/*E. reichenowi* clade. Although Honma et al. (2007) also found the clade of *E. gruis* and *E. reichenowi* as sister taxa to the remaining eimeriid coccidian with Stieda bodies, in this assessment no *Eimeria* sp. from poikilothermic hosts was included. Clearly, additional data regarding all available taxa will be needed to resolve this aspect of eimeriid relationships. Finally, this is also an atypical example of *Eimeria* sp. detection, i.e., fecal material is almost always used to amplify and sequence the DNA of these parasites (Motriuk-Smith et al., 2009; Power et al., 2009). Because they autotomize naturally and typically regenerate, tail samples are widely available in museum collections and stored in ethanol for genetic analyses of the lizards. The present study shows that not only *Hepatozoon* and *Sarcocystis* species, but also *Eimeria* spp., can be detected from these tissues. In the present study, both blood and tissue samples could be used to detect parasites. However, it is not yet clear if 1 source of DNA will allow more accurate detection of parasites at low levels of parasitemia. This is something that warrants further investigation.

Although molecular techniques are now standard tools for monitoring parasite populations in humans and domestic animals, their use in wild animal populations has until recently been limited. However, determining parasite distribution is crucial for conservation biologists, especially in species whose existences are undergoing challenge. The survey performed on *Podarcis* spp. indicates that various apicomplexan parasites can be detected with the use of tail tissue samples that are often collected for other purposes. At the same time, by sequencing the 18S rRNA gene, new

insights into the evolutionary history of the parasites can be obtained. Depending on the primers used, different apicomplexans will be detected, i.e., the cases of dual infection with species of *Hepatozoon* and *Sarcocystis* in *P. lilfordi* clearly highlight this point. There is a need, therefore, for further assessments of possible parasites using additional molecular markers so that a clearer picture of the spectrum of parasites infecting these species can be obtained.

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