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# Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from the western Mediterranean

**João P.M.C. Maia**[1,2], **Ana Perera**[1] and **D. James Harris**[1]

[1]CIBIO-UP, Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal;

[2]Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre FC4, 4169-007 Porto, Portugal

**Abstract:** The genus *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) is composed of intracellular haemogregarine parasites that are widely distributed among all tetrapod groups. The present study combines microscopic and molecular data on haemogregarine parasites from lizards in the western Mediterranean. We screened tissue samples and examined blood smears for the presence of species of *Hepatozoon* from four lizards, namely *Algyroides marchi* Valverde, endemic to Southeast Spain, *Podarcis bocagei* Seoane from Spain and Portugal, *P. hispanica* Steindachner from Spain, and *P. lilfordi* Günther from Cabrera, Balearic Islands (Spain). Our results show that prevalence and intensity of *Hepatozoon* parasites vary between and within lizard species from different regions. *Algyroides marchi* and *P. bocagei* from Spain had the lowest values, whereas *P. hispanica* had the highest. Phylogeny based on 18S rRNA gene sequences indicates that most of the new *Hepatozoon* sequences are part of a clade exclusive from North African and Iberian lizards, except for a single *P. bocagei* isolate that is found related to another clade including isolates from other reptile host species and rodents. Interestingly, isolates from *Algyroides* form a distinct monophyletic subgroup, which could be a signal of strict host-specificity within this host genus.

**Keywords:** Apicomplexa, haemogregarine, 18S rRNA, phylogeny, tissue, blood smears

The study of parasites is important not only in terms of understanding biodiversity as a whole but also for seeking answers to more complex questions related to host-specificity and co-evolution (Poulin and Mouillot 2005, Paterson and Piertney 2011). However, there is a bias within the study of parasites, with most research focusing on parasites that are considered of great veterinary, medical and public health importance. Hence, most information available is dedicated to parasites affecting domestic animals rather than wild species, with parasites infecting groups such as reptiles being still poorly studied.

Haemogregarines represent an important parasite group with veterinary importance for some groups of their hosts, such as dogs (Baneth et al. 2003), and they are one of the most common parasites found in reptiles. Haemogregarines are a group of apicomplexan (Apicomplexa, Adeleorina) intracellular parasites and four genera within this group are known to infect reptiles: *Hepatozoon* Miller, 1908, *Haemogregarina* Danilewsky, 1885, *Karyolysus* Labbé, 1894 and *Hemolivia* Petit, Landau, Baccam et Lainson, 1990 (Smith 1996, Smith and Desser 1997, Telford 2009). The genus *Hepatozoon* is the most widely distributed among reptiles and also has been reported in all other tetrapod groups (Telford 2009). Lizards of several genera, e.g., *Iberolacerta* Arribas, *Podarcis* Wagler, *Psammodromus* Fitzinger and *Timon* Tschudi, from the Iberian Peninsula have been shown to have high levels of haemogregarine infection using traditional blood smear inspection methods (Álvarez-Calvo 1975, Amo et al. 2004, 2005a, b, Jovani et al. 2004, Martín et al. 2007, Roca and Galdón 2010, Sacchi et al. 2011). This makes lizards from this region potentially good models for studying host-parasite interactions. In contrast, to our knowledge there are no studies on the prevalence and intensity of *Hepatozoon* in species of *Algyroides* Bibron et Bory de Saint-Vicent from the Iberian Peninsula.

Molecular data of *Hepatozoon* parasites in public databases, such as GenBank, are also mainly biased towards socio-economically important species, especially domestic and wild mammals. This is also the case of the Iberian Peninsula, for which multiple *Hepatozoon* parasites have been found in mammals and their definitive hosts, i.e. arthropods (Garcia et al. 1990, Criado-Fornelio et al. 2003, 2006, 2007, 2009, Ortuno et al. 2008, Tabar et al. 2008, Yabsley et al. 2008, Lledó et al. 2010). Although *Hepatozoon* sequences from reptiles are scarce in GenBank, there are a few from snakes (Ujvari et al. 2004, Harris et al. 2011, Moço et al. 2012, Tomé et al. 2012) and liz-

Address for correspondence: J.P.M.C. Maia, CIBIO-UP, Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto, Rua Padre Armando Quintas, 7, Campus Agrário de Vairão, 4485-661 Vairão, Portugal. Phone: +351 252660411; Fax: +351 252661780; E-mail: jpmaiapt@gmail.com

ards, with records from the Seychelles (Harris et al. 2011), North Africa (Maia et al. 2011) and the Iberian Peninsula and Balearic Islands (Harris et al. 2012). These studies have shown that 18S rRNA gene primers successfully amplify *Hepatozoon* from tail tips collected from lizards, highlighting the utility of this method for screening and providing new insights into parasite phylogeny.

*Hepatozoon* currently includes more than 300 species (Smith 1996) with variable morphological characteristics, diverse life histories (Smith and Desser 1997) and a wide range of host species. Nevertheless, many of these species have been described solely on morphological characteristics of the parasites found in different host species, which may be problematic if host specificity is low (Mathew et al. 2000). Based on diverse morphological and developmental characteristics, Smith and Desser (1997) suggested that the genus should be partitioned into two genera of adeleorin parasites, which was supported by a recent study on the phylogeny of the adeleorinid coccidia, which has indicated paraphyly of *Hepatozoon* (Barta et al. 2012).

In addition, Barta et al. (2012) showed that these parasites seem to have a relatively high level of host-parasite specificity with their definitive hosts (invertebrates) rather than intermediate hosts (vertebrates), in particular with the separation of parasites from leeches as a distinct clade from those from arthropods. Although four main lineages based on 18S rRNA gene sequences have been described in lizards so far (Maia et al. 2011), the phylogenetic relationships within *Hepatozoon* seem to be complex and remain largely unresolved.

The aim of this study is to increase the knowledge of haemogregarines in lizards from the Iberian Peninsula and Cabrera Island by combining both molecular and morphological methods, and to relate this information with the current literature on *Hepatozoon*. When possible, parasite load was quantified from positive samples.

## MATERIALS AND METHODS

### Sample collection

Blood smears and tissue samples were collected from lizards from different localities from the Iberian Peninsula: Gião and Viana do Castelo (Portugal), Alba de Tormes, Tanes, Palacios de Compludo, Albacete, Jaén, Rambla los Vaquerizos and Pedro Andrés (all from Spain) and Cabrera (Balearic Islands, Spain). Species were identified by experienced herpetologists in the field, body size (snout-vent length, SVL, to the nearest millimetre) and sex (based on colour pattern and the existence of developed femoral pores) of the host were also registered. After sample collection, animals were released at the capture site.

Tissue samples were preserved for molecular analysis (tail tips containing blood stored in 96% ethanol) and used to screen the presence of *Hepatozoon* parasites. When enough blood was available from the autotomized tail, blood smears were made, then air-dried, fixed with methanol and stained with Giemsa (Telford 2009). A total of 104 tissue samples from two species were collected (Table 1). Additionally, 19 *Podarcis hispanica* Steindachner type 1b samples from Spain (June 2009) and ten *P. lilfordi* Günther samples from Cabrera Island (September 2010) included in Harris et al. (2012) were also used in this study for microscopic examination and further molecular and phylogenetic analyses.

### DNA extraction, amplification and sequencing

DNA was extracted from tissue using standard High Salt methods (Sambrook et al. 1989). Detection of parasites was made using PCR reactions with the primers HEMO1 and HEMO2 (Perkins and Keller 2001), targeting part of the 18S rRNA gene region, and using the primers HepF300 and HepR900 (Ujvari et al. 2004), targeting another partially overlapping part of the 18S rRNA gene region. Conditions of the PCR are detailed in Harris et al. (2011). Briefly, PCR cycling for the HEMO primers consisted of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min (35 cycles), whereas for the Hep primers annealing temperature was 60 °C. A total of 133 samples were analysed using PCR, with negative and positive controls 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. Sequences were deposited in GenBank under the accession numbers JX531906 to JX531973.

Samples from *P. hispanica* from Spain and *P. lilfordi* from Cabrera Island were used in a previous study using a shorter fragment of the *Hepatozoon* 18S rRNA gene (Harris et al. 2012). In this study we amplified and sequenced the missing region using the Hep primers to be able to compare the full 1401 bp length (see Table 1).

### Phylogenetic analysis

Consensus sequences for each individual were created by combining the sequences of the two partially overlapping 18S rRNA gene regions and analysed using Geneious 5.6.2 (Drummond et al. 2012). Some sequences had heterozygous positions, which could indicate the existence of different *Hepatozoon* isolates in the same individual, or variation within the multiple copies of the 18S rRNA gene within the same parasite. These were given the corresponding IUPAC code letter.

We conducted two separate analyses, one containing a longer 18S rRNA gene segment (1401 bp, combining sequences obtained with Hep and HEMO primers) (Harris et al. 2011, Maia et al. 2011) and the other containing a shorter 18S rRNA gene segment (562 bp, obtained with Hep primers) in order to compare their phylogenetic resolution and because many previously published sequences were available only for the shorter fragment. Since not all samples worked for both fragments, a total of 49 new parasite sequences were obtained for both fragments, whereas 68 parasite sequences were obtained only for the shorter segment.

Sequences were blasted in GenBank and all matched known *Hepatozoon* sequences. *Hepatozoon* sequences available were downloaded from GenBank and those representing different *Hepatozoon* haplotypes for the major clades were included in the phylogenetic analysis (see Fig. 2). In addition, the newly available sequences from other adeleorinid parasites were also included (HQ224961 *Babesiosoma stableri* Schmittner et McGhee, 1961, HQ224957 and HQ224958 *Dactylosoma ranarum* Labbé, 1894, and HQ224959 *Haemogregarina balli* Paterson et Desser, 1976). Although there is a sequence from *Hemolivia mariae* Smallridge et Paperna, 1997 available in GenBank (JN211118), it was not included in the analysis because it only

**Table 1.** Summary of the samples analysed in this study.[1]

| Host | Country | Sample size (males/females) | Molecular screening | | | | Microscopic examination | |
|---|---|---|---|---|---|---|---|---|
| | | | N positives (males/females) | Prevalence (%) | HEMO | Hep | Positives analysed (males/females) | Mean intensity % (min–max) |
| *Algyroides marchi* | Spain | 66 | 29 | 44 | 19 | 29 | 7 (3/4) | 0.57 (0.17–2.05) |
| *Podarcis bocagei* | Portugal | 26 (14/12) | 17 (10/7) | 65 | 13 | 17 | 6 (3/3) | 2.95 (0.10–6.88) |
| *Podarcis bocagei* | Spain | 12 (7/5) | 5 (2/3) | 42 | 4 | 5 | 5 (2/3) | 0.49 (0.25–1.14) |
| *Podarcis hispanica* type1b * | Spain | 19 (10/9) | 19 (10/9) | 100 | 17 | 19 | 19 (10/9) | 2.52 (0.30–11.55) |
| *Podarcis lilfordi* * | Cabrera | 10 (3/7) | 7 (3/4) | 70 | 7 | 2** | 7 (2/5) | 0.97 (0.42–2.77) |
| | | 133 | 77 | 58 | 60 | 72 | 44 (20/24) | 1.79 (0.10–11.55) |

[1] Prevalence is based on positive PCR results; * samples from Harris et al. (2012); ** as described by Harris et al. (2012); HEP primers preferentially amplified *Sarcocystis* in double infections.

partially overlapped with the short fragment used here. The final alignments contained 113 *Hepatozoon* sequences that were 1401 bp long, whereas the second analysis contained 151 *Hepatozoon* sequences that were 562 bp long. Sequences were aligned with the ClustalW algorithm using default parameters implemented in Geneious 5.6.2 and checked by eye.

Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships, using the software PhyML 3.0 (Guindon et al. 2010). Support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 500 replicates. The AIC criterion conducted in Modeltest 3.06 (Posada and Crandall 1998) was used to choose the model of evolution and the parameters employed (TVM+G for the shorter fragment, TVM+I+G for the longer fragment).

Bayesian 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 $10 \times 10^6$ generations, saving one tree each 1000 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). *Adelina* Hesse, 1911 was used as an outgroup for rooting the phylogenetic tree (Morrison 2009). Pairwise uncorrelated differences (p-distance) were estimated using MEGA 5.05 (Tamura et al. 2011).

In order to facilitate the analysis of the phylogenetic relationships within lineage 2 isolates, a network was made using only the longer sequences from this lineage. The network was produced using a Median-Joining analysis with default parameters in the software Network 4.6.1.0 (Bandelt et al. 1999).

**Microscopic examination**

Blood smears were examined using an Olympus CX41 microscope with an in-built digital camera (SC30). Several photomicrographs per slide were taken at 400× magnification and stitched using cell^B software (basic image-acquisition and archiving software, Olympus, Germany). Intensity of infection, i.e. the percentage of infected cells within a host, was estimated as percentage based on counts of haemogregarines per 2000 cells for a total of 44 blood smears (Table 1). Counts were done using the manual cell counter plug-in available in the image processing software ImageJ ver. 1.44p (Abramoff et al. 2004). Fig. 1 shows intracellular gamonts of *Hepatozoon* infecting the four lizard species.

**Statistics**

Comparison in prevalence between sexes per locality was assessed using Fisher's Exact Test, which is more appropriate than a chi-square test in cases of $2 \times 2$ tables with low frequencies (Zar 2009). Correlation between host body size (snout-vent length) and parasitemia levels was investigated using a Spearman correlation. All analyses were performed using R (R Development Core Team 2009).

## RESULTS

Of the 133 samples analysed using molecular markers, 77 were positive, which gives an overall prevalence of 58%. Primer performance differed between the two sets used in this study. Overall, the Hep primers performed better at detecting, amplifying and sequencing *Hepatozoon* than the HEMO primers, except in double infection cases (see Table 1). Prevalence of *Hepatozoon* sp. was highest in *Podarcis hispanica* (100%), followed by *P. lilfordi* (70%), *P. bocagei* Seoane from Portugal (65%) and *Algyroides marchi* Valverde (44%) with *P. bocagei* from Spain having the lowest prevalence (42%) (Table 1). There was no significant variation of prevalence between sexes (Fisher's Exact Test, in all cases $p > 0.05$). Intensity of infection, i.e. the percentage of infected cells, varied across and within host species and geographical locations. *Algyroides marchi* and *P. bocagei* from Spain showed the lowest haemogregarine intensities, whereas the highest intensities were found in *P. bocagei* from Portugal and *P. hispanica* type 1b. There was no relation between snout-vent length and intensity of infection in any of the populations analysed (Spearman Correlation, in all cases $p > 0.5$).

The two phylogenetic methodologies used to estimate phylogeny produced similar tree topologies and thus only one is presented (Fig. 2). Two main lineages were found, one composed of a single *Hepatozoon* isolate from the lacertid *P. bocagei* from Portugal (lineage 1) and other lineage of the remaining Iberian and Cabrera *Hepatozoon* isolates (lineage 2) (see Fig. 2). Lineage 1 is closely related to one of the lineages found in North Africa that also infects a lacertid host (*Timon tangitanus* Boulenger). In contrast, all other *Hepatozoon* isolates (from *A. marchi*, *P. hispanica*, *P. bocagei* and *P. lilfordi*) are closely related to a generally unresolved *Hepatozoon* lineage found

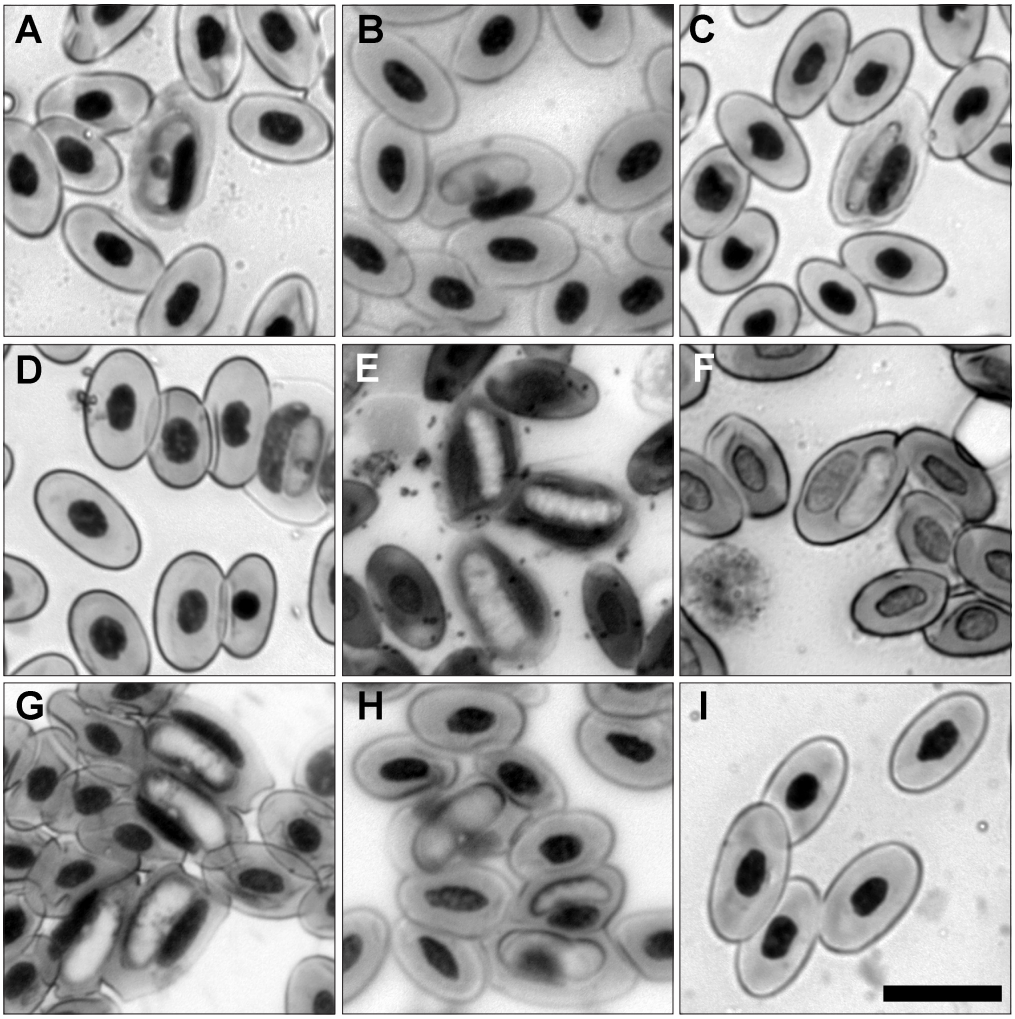


**Fig. 1.** *Hepatozoon* parasites infecting erythrocytes from *Algyroides marchi* from Spain (DB9365AmSP and DB9379AmSP) (**A**, **B**), *Podarcis lilfordi* from Cabrera Island (DB10319PlCA and DB10553PlCA) (**C**, **D**), *P. bocagei* from Portugal (3251PbPO and 3284PbPO) (**E**, **F**), and *P. hispanica* from Spain (5204PhSP, 5212PhSP) (**G**, **H**). Normal, uninfected, lizard erythrocytes (from DB9171AmSP) (**I**). Scale bar = 10 µm.

in skinks (*Eumeces algeriensis* Peters and *Chalcides polylepis* Boulenger) and several lacertids from North Africa (including another *Podarcis* species, *P. vaucheri* Boulenger), and lacertids from the Iberian Peninsula and Balearic Islands (Fig. 2).

Sequence divergence between the isolate found in the lineages 1 and 2 is 1.3–1.6% (for the longer segment) and 3.1–3.3% (for the shorter segment). Within the lineage 2, all *A. marchi* isolates form a monophyletic subgroup, whereas relationships between isolates from *P. bocagei* and *P. hispanica* are not well resolved. The network of the unresolved lineage 2 shows that *Hepatozoon* parasites from *P. bocagei* and *P. hispanica* can be found in two groups, whereas those from *A. marchi* form a unique subgroup (Fig. 3).

## DISCUSSION

Our results show that both parasite prevalence and intensity vary between and within host species and their geographical locations. Overall, prevalence was high in our study, which is in accordance with the morphological studies on lizards from the same region. A microscopic survey of *Podarcis carbonelli* Pérez Mellado and *P. bocagei* from northwest Portugal showed similar prevalence of haemogregarines, 69.7% and 74.7%, respectively, although intensities were not reported (Roca and Galdón 2010). Another microscopic survey on *Podarcis muralis* Laurenti from Spain found 58.1% prevalence and intensity levels of less than 0.5% (Amo et al. 2005a).

In this study we found varying prevalence values for parasites infecting the genus *Podarcis* Wagler, with all individuals infected from one population of *P. hispanica*, whereas for *P. bocagei* from Spain less than half of the individuals analysed were infected. In fact, within *P. bocagei* from Portugal we found high prevalence (65%, n = 26) and high mean intensity levels (2.95%, n = 6), whereas in Spain prevalence and intensity values were much lower (42%, n = 12, and 0.49%, n = 5, respectively).

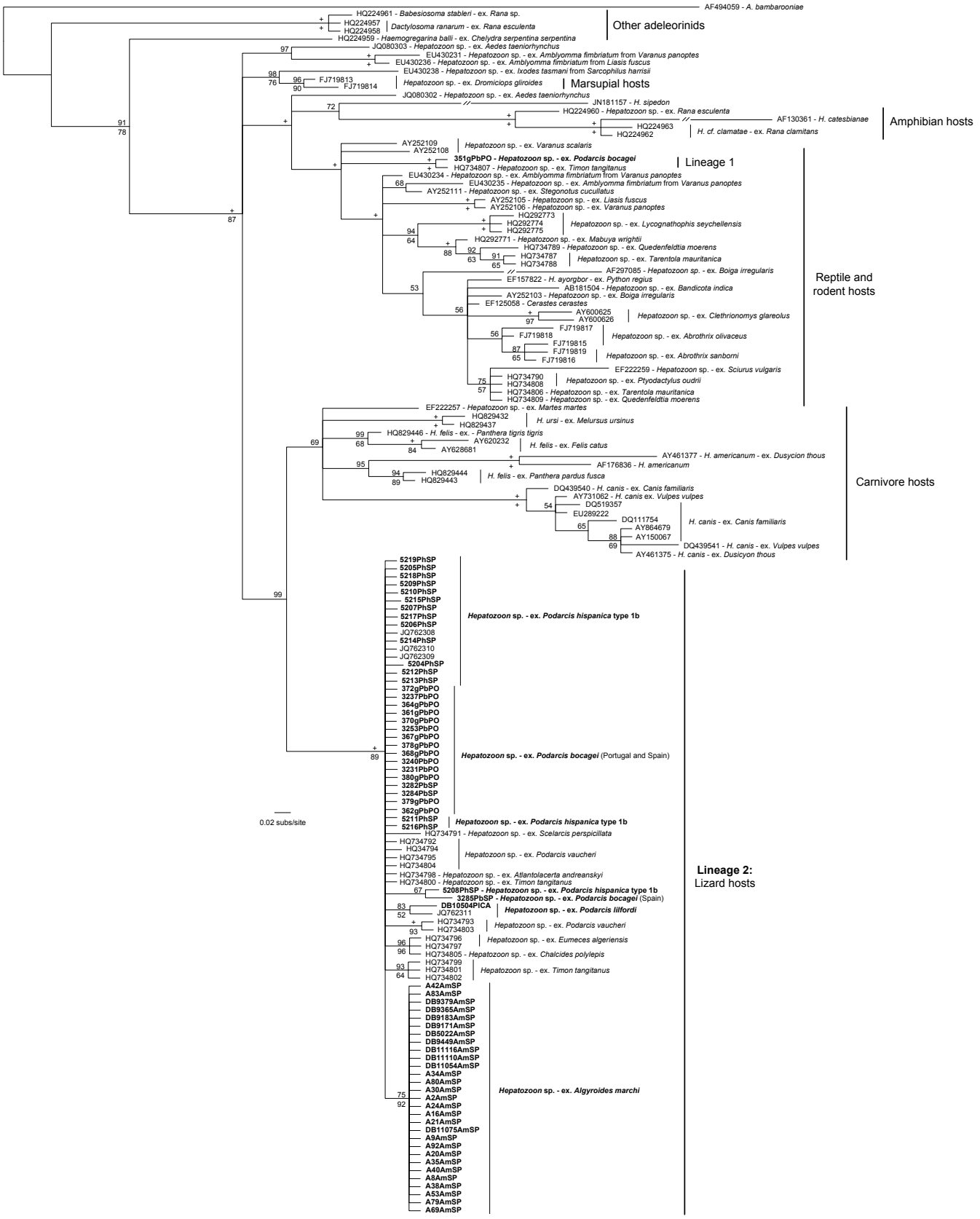


**Fig. 2.** Bayesian estimate of relationships of *Hepatozoon* species based on 562 bp 18S rRNA gene sequences. Bayesian posterior probabilities are given above the nodes and ML bootstrap values below them. When both values were 100%, this is indicated with a +. New haplotypes from this study are in bold. The branches of JN181157, AF130361 and AF297085 were shortened by 50%.

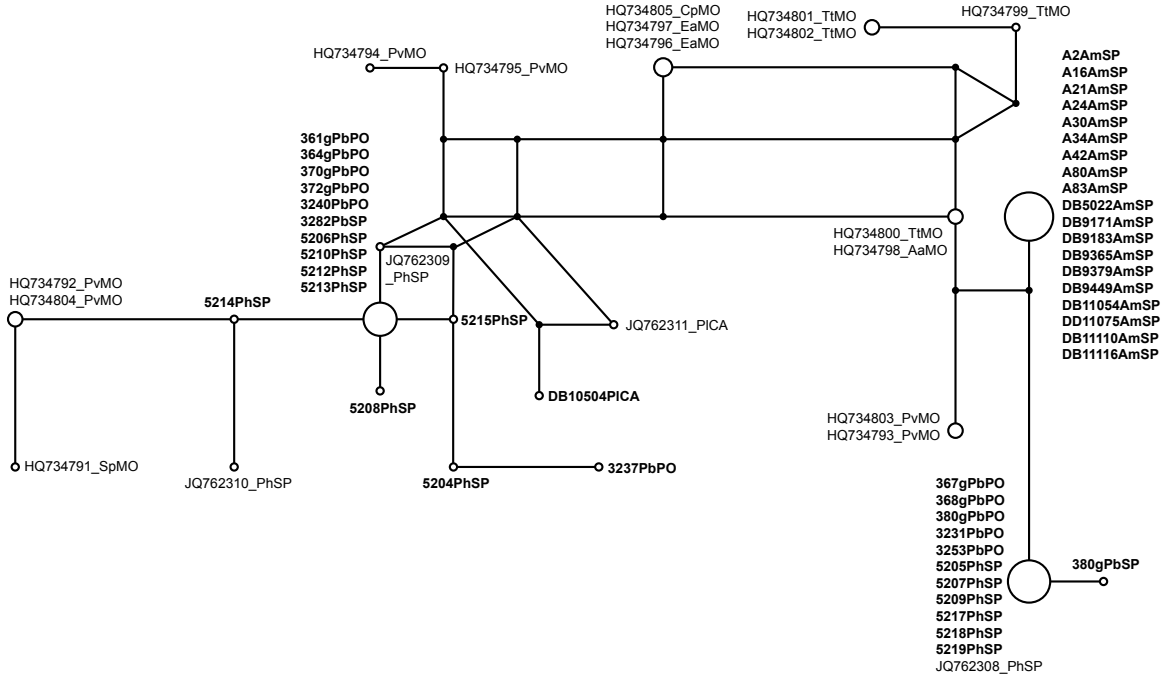


**Fig. 3.** Median-Joining Network analysis of lineage 2, using 1401 bp 18S rRNA gene sequences. New haplotypes from this study are in bold. Last four letters indicate host species initials (first two letters) and country (last two letters).

Nevertheless, since only 12 individuals were screened for *P. bocagei* in Spain, this difference could be biased by non-representative sampling.

Interestingly, mean intensity values for *P. bocagei* are similar to those of *P. muralis* reported by Amo et al. (2005a). Differences in prevalence and intensity of haemoparasites (either between or within host species) could be associated with factors such as microhabitat characteristics (Davis et al. 2012), feeding habits and seasonality. Indeed, seasonality has been shown to be associated with differences in parasite load (Salkeld and Schwarzkopf 2005, Santos et al. 2005). However, since our samples were collected during different seasons and in different years, we cannot draw conclusions regarding this. This could be investigated by conducting large-scale sampling of individuals from several species from a single location and from the same species from different locations at different seasons. Also, we still know little about the influence that these parasites may have on host health and fitness, and this could also be investigated using immunological and performance tests (Sacchi et al. 2007).

To our knowledge, this is the first assessment of the occurrence of haemogregarines in the species *Algyroides marchi*. There is only a record of haemogregarine infection in *Algyroides nigropunctatus* Duméril et Bibron (from Italy), with a mean intensity of about 0.2% (no prevalence records are provided), in a survey performed with the purpose of comparing counts and morphology of leukocytes in 12 lizards species (Sacchi et al. 2011). In our study, intensity levels in *A. marchi* were very similar to those found in its Italian relative (mean = 0.57, min.–max.: 0.17–2.05).

Our study highlights the utility of the short 18S rRNA fragment amplified using the Hep set of primers for large-scale molecular surveys of *Hepatozoon* parasites, since the estimate of phylogenetic relationships is similar to those retrieved using the longer fragment. In addition, Hep primers usually perform better at detecting infections and normally yield better quality sequences. Although a larger fragment provides more genetic information and acts as a safety net against contamination, because it requires two separate PCRs, the sole use of Hep primers provides similar estimates of phylogenetic relationships at lower cost. Nonetheless, as realized recently, Hep primers have the potential of amplifying DNA of other apicomplexan parasites and in double infection some primers may preferentially amplify other apicomplexans, such as *Eimeria* Schneider, 1875 and *Sarcocystis* Lankester, 1882 coccidia (Harris et al. 2012). Hence, in these situations samples should also be tested with the HEMO primers to check for the presence of haemogregarines, since these are more specific primers.

The phylogenetic relationships estimated for *Hepatozoon* parasites remain similar to those previously described (Maia et al. 2011, Harris et al. 2012), despite the addition of new sequences for the 18S rRNA gene in this study. However, the present phylogenetic analysis increases the known geographical distribution of this group and the number of host species infected by these parasites. One lineage from North Africa, previously known only from the lacertid host *Timon tangitanus*, appears to be related with one of the new isolates from *P. bocagei*, whereas all the other new isolates fall in a group with another lineage from North Africa found in lacertids and skinks.

The occurrence of two very distinct genetic lineages of *Hepatozoon* isolates from the same host species demonstrates that a single host species can harbour genetically distinct isolates. Since the 18S rRNA is a slow evolving gene, the high sequence divergence found between the lineages 1 and 2 could indicate that these represent different, unrelated species of *Hepatozoon*. Unfortunately, no blood smear was made at the time of collection for the unique sample from lineage 1 from *P. bocagei* and thus it is not possible to couple microscopy with phylogeny for this lineage.

Still, this result emphasizes that *Hepatozoon* may have low host-specificity (Telford et al. 2001), at least concerning some intermediate host species such as *P. bocagei*. Thus, the transmission cycle should be fully studied in the future, by collecting from a single location (with known parasitic infections) both the vertebrate host and the potential vectors that are the definitive hosts (arthropods which are often found attached to the intermediate host, such as ticks and mites, but also mosquitoes and reduviid bugs – Telford 2009). This is especially important since relatively high degree of host-parasite association of various species of Apicomplexa with their definitive hosts have been observed recently (Barta et al. 2012).

In contrast, the fact that *Hepatozoon* isolates from *Algyroides* form a monophyletic subgroup within the lineage 2 demonstrates some degree of strict intermediate host specificity. Finally, the phylogenetic relationships between the isolates found in *P. bocagei* and *P. hispanica* remain largely unresolved and given that the hosts are closely related species (together forming a species complex – see Pinho et al. 2008) it is possible that these hosts share the same parasite communities. Nonetheless, further research should be conducted using faster evolving genes, to obtain a better resolution of this lineage.

By including other adeleorinid sequences in our phylogeny, we demonstrate in accordance with Barta et al. (2012) that these are clearly distinct from our *Hepatozoon* sequences, thus discarding the possibility of the lineage 1 being from other adeleorinids, a possibility that could not previously be rejected (Maia et al. 2011). Support levels for the major groups within *Hepatozoon* are also low, further indicating that more data are necessary prior to any taxonomic changes being appropriate.

Finally, our phylogeny also presents some incongruences in sequences deposited in GenBank. For example, the sequence identified as the snake *Cerastes cerastes* Linnaeus in GenBank (EF125058) is actually a *Hepatozoon* sp., presumably accidentally amplified instead of the host. This kind of erroneous amplification of parasites instead of hosts, and subsequent mislabelling in databases, has also been recorded for flatworms (Heneberg 2012), and further highlights the need for care when comparisons are made with sequences from GenBank.

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