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Molecular identification of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) haemoparasites in *Podarcis muralis* lizards from northern Italy and detection of conserved motifs in the 18S rRNA gene

Simona Panelli, Marianna Bassi and Enrica Capelli

Department of Earth and Environmental Sciences, Section of Animal Biology, Laboratory of Immunology and Genetic Analyses and Centre for Health Technologies (CHT)/University of Pavia, Via Taramelli 24, 27100 Pavia, Italy

(Corresponding author: Enrica Capelli, enrica.capelli@unipv.it)
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ABSTRACT: This study applies a non-invasive molecular test on common wall lizards (*Podarcis muralis*) collected in Northern Italy in order to i) identify protozoan blood parasites using primers targeting a portion of haemogregarine 18S rRNA; ii) perform a detailed bioinformatic and phylogenetic analysis of amplicons in a context where sequence analyses data are very scarce. Indeed the corresponding phylum (Apicomplexa) remains the poorest-studied animal group in spite of its significance for reptile ecology and evolution.
A single genus, i.e., *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) and an identical infecting genotype were identified in all positive hosts.
Bioinformatic analyses identified highly conserved sequence patterns, some of which known to be involved in the host-parasite cross-talk. Phylogenetic analyses evidenced a limited host specificity, in accord with existing data.
This paper provides the first *Hepatozoon* sequence from *P. muralis* and one of the few insights into the molecular parasitology, sequence analysis and phylogenesis of haemogregarine parasites.

Keywords: haemoparasites, lizards, *Hepatozoon*, *Podarcis* spp., bioinformatics, conserved sequence motifs, untranslated regions.

INTRODUCTION

Protozoan blood parasites in reptiles are known to act as relevant selective agents affecting host populations at the level of ecological attributes, biology and evolution, with effects on growth, sexual selection and reproduction, spatial distribution. However, the characterization of these parasites and of their relation with hosts remain poorly understood (Schall *et al.* 1990; Martin *et al.* 2008).

These parasites belong to the phylum Apicomplexa, that comprises widely studied genera as *Plasmodium*, *Babesia* and *Toxoplasma*. Apart from these medically relevant exceptions, the phylum remains the poorest-studied group of all animals, with only about 0.1% of the estimated species number described to date (Morrison *et al.* 2009), a few sequences deposited in public databases and scarce molecular and sequence analysis data available. With respect to reptile parasitology, the most important group within the phylum is constituted by haemogregarine (Apicomplexa, Adeleorina), also causative agents of severe zoonoses in mammals, to which they are transmitted by arthropodes, representing their definitive host (Baneth *et al.* 2003). Four genera within this group are known to infect reptiles: *Hepatozoon*, which represents the most relevant genus with respect to the zoonotic infections (Vincent-Johnson *et al.* 1997), *Haemogregarina*, *Karyolysus* and *Hemolivia* Smith 1996; Smith and Desser, 1997).

The common wall lizard, *Podarcis muralis*, is an European small lacertid lizard, highly adaptable to various environments and urban settings. Its natural range spans mainland Europe, including the northern area of the Iberian peninsula, southern Belgium, and the Netherlands (Amo *et al.* 2005). In Italy, *P. muralis* commonly inhabits northern and central regions. Blood parasites frequently occur in this species and have been found to affect health and body conditions in both wild and captive populations (Amo *et al.* 2005; Martin *et al.*, 2008; Galeotti *et al.* 2010). However, studies on blood parasites in this species not rarely lack further characterization (e.g., phylogenetic, bioinformatic) and rely on traditional blood smear inspection methods.

The present study applies a non-invasive molecular test on micro-quantities of peripheral blood of *P. muralis* lizards in order to: i) identify blood parasites using primers amplifying ~900 bp in the 3' portion of the 18S rRNA locus of haemogregarine species; ii) sequence the amplicons in both directions in order to perform detailed bioinformatic and phylogenetic analyses.

MATERIALS AND METHODS

A. Ethical statement

The study was carried out in conformity with the current Italian laws for lizard collection and detention (Aut. Prot. DPN-2009-0016034).

B. Sample collection

Thirteen *P. muralis* sexually mature lizards (>50 mm snout-vent length, SVL, see Sacchi *et al.* 2011) were captured by noosing in April 2012 in the surroundings of Pavia (Lombardy, northern Italy). About 25 µl blood was collected from the postorbital sinus into heparinized capillary tubes and stored at -20°C till DNA extraction.

C. DNA extraction, amplification and sequencing

DNA was extracted using Quick-gDNA Microprep kit (Zymo Research corp., Irvine, CA), a commercial kit specifically designed for micro-quantities of blood, serum or plasma, especially for forensic applications. DNA concentration and purity were assessed using the fluorimetric method (Qubit 2.0 Fluorometer, Thermo Fisher Scientific, Waltman, MA). Samples were stored at -20°C and subsequently used to amplify part of the 18S rRNA locus using the haemogregarine-specific primers HEMO1 and HEMO2 (Perkins *et al.* 2001). PCR reactions were carried out in 12.5 µl containing 40 ng genomic DNA, 7 µl Gotaq Green Master Mix (Promega corp., Madison, WI) supplying the reaction buffer, $MgCl_2$ and Taq DNA polymerase, 1 µM of each primer, 2.5 µl of nuclease-free water. Cycling parameters were: initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 30 s, 48°C for 30s, 72°C for 1 min, and final extension at 72°C for 10 min. PCR was performed on a MJ Mini Personal thermal cycler (Promega corp., Madison, WI). Negative and positive controls were run at each reaction. Amplicons were purifed by a commercial kit (Machery Nagel, Duren, Germany) and sequenced in both directions using a 310 ABI PRISM automated DNA sequencer (Thermo Fisher Scientific, Waltman, MA).

D. Bioinformatic analysis

Electropherograms were visually inspected and manually edited for quality using Bioedit (http://www.mbio.ncsu.edu/ BioEdit/BioEdit.html) (Hall 1999). A consensus sequence for each individual was created by combining the two partially overlapping sequences obtained using each primer. The retrieved parasite sequence, ~ 820 bp long, resulted unique for all positive lizard samples. Similarity searches were performed by means of the basic alignment search tool program within the GenBank database (www.ncbi.nlm.nih.gov/BLAST), also using the available specialized protozoa genomic pages. Specialized protozoa database, specifically ProtozoaDB 2.0 (http://protozoadb.biowebdb.org/) and Gene DB (http://www.genedb.org/Homepage) were also interroged. Repetitive DNA elements were searched by means of the Repeat Masker server (www.repeatmasker.org). Finally, functional and regulatory elements as gene localization and regulation motifs were retrieved using UTRscan algorithm to search the UTR-site database (http://itbtools.ba.itb.cnr.it/utrscan) (Grillo *et al.* 2010).

E. Phylogenetic analysis

The parasite sequence evidenced in the analyzed lizards, identified as *Hepatozoon* spp, was aligned with 18S rRNA entries downloaded from the GenBank, and referring either to *Hepatozoon* parasites isolated from various hosts (lizards, snakes, rodents and carnivores) and from various geographical locations. The Mega6 software (Kumar *et al.* 2004) was used for aligning sequences by means of the ClustalW algorithm. For the phylogenetic analysis, two methodologies were used. First, a maximum likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was conducted to estimate evolutionary relationships and patterns of genetic diversity using PhyML 3.0 (Guindon *et al.* 2010) (http://www.atgc-montpellier.fr/phyml/). Support for nodes was estimated by using the bootstrap technique (Felsenstein, 1985) with 1000 replicates. The model of evolution employed was chosen using the AIC criteria conducted in jModelTest 0.1.1 (Posada 2008) (http://darwin.uvigo.es.). Finally, Bayesian analysis was reconstructed using the inference method implemented in MrBayes (v3.2.3) (Ronquist and Huelsenbeck 2003). The analysis was run for 100,000 generations saving one tree every 100 generations. The log-likelihood values of the sample points were plotted against the generation time and all trees prior to stationary were discarded as "burn-in" samples. Remaining trees were combined in a 50% majority rule consensus tree. The species used as outgroups were other adeleorinid parasites, chosen following Maia *et al.* (2012) and Haklová-Kočíková *et al.* (2014).

RESULTS AND DISCUSSION

A. Molecular analysis

The yields of DNA extracted from peripheral blood were in the range of 22-40 ng/µl, with total eluates of 20 µl. Amplicons were reproducibly obtained from four over thirteen samples, depicting a prevalence of about 31%. Fig. 1 shows a representative amplification of positive lizards. PCR products were then sequenced directly in both directions. Differently from what observed by other researchers (Maia *et al.* 2012; Haklová-Kočíková *et al.* 2014) we were able to produce sequences of satisfying quality, about 820 bp long, from products obtained with HEMO1/HEMO2, without the need of using other primers, as the Hep couple, which produces shorter amplicons.

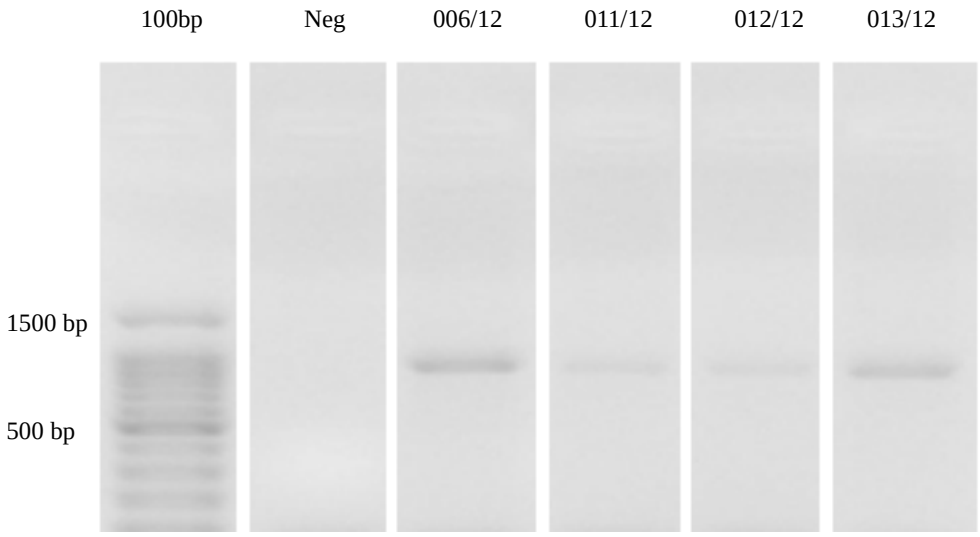

Fig. 1. Representative PCR amplification of positive lizards. DNA extracted from peripheral blood was amplified with haemogregarine primers HEMO1/HEMO2 in the described conditions. The molecular weight marker is a 100 bp ladder.

The four parasitic sequences resulted identical and GenBank searches allowed to identify them as *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina): the first sequence report of an *Hepatozoon* parasite from *P. muralis* to our knowledge. The new sequence was deposited under the Acc. No. KU529653.

B. Bioinformatic analysis and search for conserved motifs

In general, the few molecular data for *Hepatozoon* haemoparasites in public databases are strongly biased towards socio-economically important species. For reptiles, there are only a few reports (e.g., Harris *et al.* 2011; Maia et al. 2011; Harris *et al.* 2012) and, in general, a lack of sequence analysis data. This prompted us to further characterize and annotate our sequence.

Similarity searches performed on KU529653 using specialized genomic pages at NCBI, as well as protozoa database, identified the presence of long sequence strings strongly conserved in other Apicomplexa species. The longest homology was identified for *Plasmodium vivax* Sal1 (Acc. No. NC_009910): a reconstructed genomic scaffold (NW_001852096.1) exhibited 86% homology with ntt 6-440 of KU529653. Due to its length (435 bp), this conserved region was examined in more details by means of further BLAST searches, using the general nucleotide database. The sequence confirmed to be highly conserved and produced hits (99% homology) with 18S rRNA genes across large phylogenetic distances, among which many mammals. It was even recognized as a human repetitive sequence by the Repeat Masker server.

We thus decided to look for the presence of functional elements. The whole KU529653 sequence was analysed by using the UTRscan software, that retrieves regulatory sequences ascribable to the 5' and 3' untranslated (UTR) regions of eukaryotic mRNAs (Grillo *et al. 2*010).

The search, in a ribosomal gene, of mRNAs regulatory elements was suggested by recent evidences. Pánek *et al.* (2013) observed regions of highly specific, statistically significant, complementarity between 18S rRNA genes and regulatory elements of mRNAs. Such regions, that resulted specifically restricted to the the 18S gene among the ribosomal cluster, appeared strongly conserved among fourteen distant eukaryotic species, from the protozoan *P. vivax* to man. Authors propose that they could be involved in rRNA-mRNA interactions for regulating gene expression at the level of protein synthesis.

Results for the search are presented in Table 1. The whole KU529653 18S sequence was found to be scattered with sequences showing regulatory function on gene expression: five putative upstream open reading frames (uORF) and three conserved motifs of 3' untranslated (UTR) regions of eukaryotic mRNAs. The former are generally found in 5' UTR regions of mRNAs and trigger mRNA decay or regulate translation (Barbosa *et al.* 2013); the latter act on mRNA stability through post-transcriptional mechanisms for a rapid response to biological and environmental cues (Pesole *et al.* 2001).

Table 1: Regulatory motifs in the sequenced region of Hepatozoon 18S rRNA (KU529653). Hits to gene regulatory motifs obtained through UTRscan searches of KU529653. Abbreviations: uORF: upstream open reading frame; MBE: Musashi binding element; ADH_DRE: alchohol dehydrogenase down-regulation control element.

Element	Position in KU529653	Known function	Ref.
uORF	63-128	Regulation of mRNA translation	(Barbosa *et al.* 2012) (Gogarten and Hilario 2006)
MBE	119-123	Temporal regulation of mRNA translation during complex processes as cell cycle regulation	(MacNicol *et al.* 2011)
uORF	138-293	Regulation of mRNA translation	(Barbosa *et al.* 2012) (Gogarten and Hilario 2006)
ADH_DRE	233-240	Down-regulation element at the 3'UTR of alcohol dehydrogenase mRNA	(Parsch *et al.* 2000)
uORF	370-492	Regulation of mRNA translation	(Barbosa *et al.* 2012) (Gogarten and Hilario 2006)
K-BOX	540-547	3' UTR down-regulation element	(Lai *et al.* 1998)
uORF	545-667	Regulation of mRNA translation	(Barbosa *et al.* 2012) (Gogarten and Hilario 2006)
uORF	688-774	Regulation of mRNA translation	(Barbosa *et al.* 2012) (Gogarten and Hilario 2006)

Our results are also in line with the already reported presence, in protozoan genes, of 3' UTR motifs typical of higher organisms. In detail: Catania and Lynch (2010) have reported the presence of mammalian 3' UTR regulatory sequences in the genome of the ciliate *Paramencium tetraurelia*, with a pronunced association with genes related to the ribosome and protein translation.

Finally, all the listed motifs (uORFs, MBE, ADH_DRE and K-BOX) have been recently detected as common patterns in the transcriptome of the bovine and feline genotypes of the flagellate protozoan parasite *Tritrichomonas foetus* (Morin-Adeline *et al.* 2014). Authors discuss in particular the presence of uORFs, already detected in rRNA genes (Tran *et al.* 2008) and well-represented in the *Plasmodium* genome, where they are and known to regulate virulence-associated traits (Amulic *et al.* 2009).

C. Phylogenetic analysis

Our data are completed by a phylogenetic analysis. To clarify the relationships of the retrieved *Hepatozoon* 18S rRNA sequence with known clades of the same genus, *Hepatozoon* sequences representing different parasitic haplotypes were downloaded from the Genbank. These sequences referred either to *Podarcis* spp. and to other hosts (lizards, snakes, rodents, carnivores). Table 2 lists the Accession Numbers of the sequences included in the phylogenetic analysis, as well as all the available additional information for each of them (e.g.: which host? Which site of sampling?). The two used phylogenetic methodologies, i.e., Bayesian method and Maximum Likelihood, gave the same overall estimate of phylogenetic patterns and produced a similar tree topology (Fig. 2). The *Hepatozoon* sequence from the Italian *P. muralis* formed part of a cluster composed by other parasitic lineages identified in lizards from the Iberian peninsula and Balearics. These lizards belong to the genera *Podarcis* and *Algyroides*. The latter, in particular, is represented by four *Algyroides marchi* individuals, captured in four different Spanish sites. The *Hepatozoon* sequence isolated from these four lizards is unique and 100% homologous to the *P. muralis* Italian lineage. In other words, the same parasitic haplotype is infecting two lizard genera. This is in line with previous reports (Maia *et al.* 2011) and confirms the already hypothesized scenario of *Hepatozoon* as a not host-specific parasite, easily switching between hosts (Maia *et al.* 2011). This paper also concludes that *Hepatozoon* infections, differently from what previously proposed (Vilcins *et al.* 2009), show no clear relation with host ecology, as the same isolate is retrievable in completely different geographical contexts. Even if limited to one parasite lineage in a single lacertid species, our data are go in the same direction.

Finally, sequences from other hosts resolve in coherence with what already published on the phylogenesis of these parasites, e.g., HQ734807 (*Hepatozoon* ex *Timon tangitanus*, Morocco) forms a separate clade with JX531921, (*Hepatozoon* ex *P. bocagei*, Portugal) (Maia *et al.* 2014). In general, our analysis confirms the existence of divergent lineages of *Hepatozoon* parasites retrievable in a same sampling locality and/or infecting the same host species (Maia *et al.* 2011).

Table 2: Sequences included in the phylogenetic analysis. Accession Numbers of *Hepatozoon* spp. 18S rRNA sequences aligned with the parasitic sequence retrieved in wall lizards from Lombardy (northern Italy). The Table also reports the site of sampling of the host.

GenBank Acc. No.	Haemoparasite	Host and site of sampling[1]
Out groups		
HQ224957	*Dactylosoma ranarum*	*Rana esculenta*
HQ224959	*Haemogregarina balli*	*Chelidra serpentina serpentina*
***Hepatozoon* ex *Podarcis* spp. (Spain and Portugal)**		
JX531906	*Hepatozoon* sp	*Podarcis hispanica*, Alba de Tormes, Spain
JX531916	*Hepatozoon* sp.	*Podarcis hispanica*, Alba de Tormes, Spain
JX531920	*Hepatozoon* sp.	*Podarcis lilfordi*, Cabrera, Balearic Islands, Spain
JX531921	*Hepatozoon* sp.	*Podarcis bocagei*, Viana do Castelo, Portugal
JX531923	*Hepatozoon* sp.	*Podarcis bocagei*, Viana do Castelo, Portugal
JX531927	*Hepatozoon* sp.	*Podarcis bocagei*, Viana do Castelo, Portugal
***Hepatozoon sp.* ex *Algyroides marchi* (Spain and Portugal)**		
JX531933	*Hepatozoon* sp.	*Algyroides marchi, Rambla los Vaquerizos, Spain*
JX531934	*Hepatozoon* sp.	*Algyroides marchi, Pedro Andres, Spain*
JX531943	*Hepatozoon* sp.	*Algyroides marchi, Albacete, Spain*
JX531944	*Hepatozoon* sp.	*Algyroides marchi, Jaen, Spain*
Hepatozoon sp.* ex *various reptile hosts		
HQ734807	*Hepatozoon* sp.	*Timon tantitanus* (lizard), Foum Kheneg, Morocco
HQ292774	*Hepatozoon* sp.	*Lycognathophis seychellensis* (snake), Fregate, Seychelles Islands
KC342525	*Hepatozoon* sp.	*Boiga irregularis* (snake), Australia
KC696565	*Hepatozoon* sp.	*Psammophis schokari* (snake), North Africa
Hepatozoon sp.* ex *rodent hosts		
AB181504	*Hepatozoon* sp.	*Bandicota indica* (rat), Chiang Mai, Thailand
Hepatozoon sp.* ex *carnivore hosts		
AY620232	*Hepatozoon felis*	*Felis catus*, Spain
DQ439540	*Hepatozoon canis*	*Canis familiaris*, Venezuela
AY461377	*Hepatozoon americanum*	*Dusycion thous*, Brazil

[1]when available

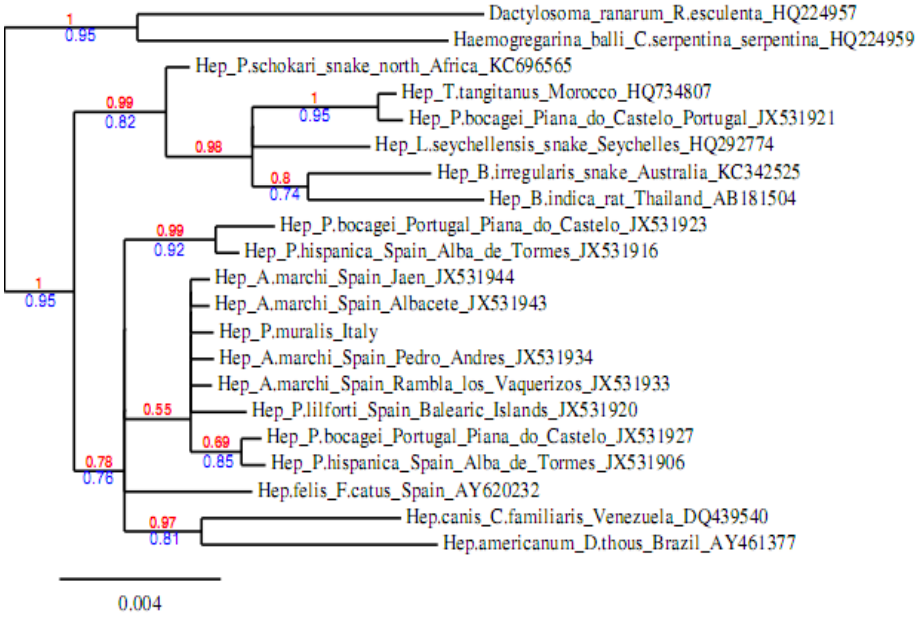

Fig. 2. Estimate of phylogenetic relationships based on 18S rRNA sequences. Bayesian posterior probabilities are given above relevant nodes and ML bootstrap support appears below them. If not explicitely stated, reptiles hosts are lizards.

CONCLUSIONS

The present analysis provides one of the few available insights into the molecular parasitology, sequence analysis and phylogenetic characterization of protozoan blood parasites of *P. muralis*. Our data report the presence, in a ribosomal gene, of putative regulatory motifs active on the regulation of gene expression and conserved across large phylogenetic distances. This is in line with emerging data on: (i) other parasitic protozoan species (*Plasmodium*, *Tritrichomonas*) and their interplay with host physiological traits, known to occur through also the same conserved regulatory motifs identified in our analysis; (ii) the presence, in eukaryotic ribosomal genes, of mRNA regulation elements as part of interactions rRNA-mRNA for regulating gene expression at the level of protein synthesis.

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