Cytochrome c oxydase I phylogenetic analysis of Haemogregarina parasites (Apicomplexa, Coccidia, Eucoccidiorida, Haemogregarinidae) confirms the presence of three distinct species within the freshwater turtles of Tunisia

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ABSTRACT

Species of *Haemogregarina* are apicomplexan blood parasites that use vertebrates as intermediate hosts. Due to limited interspecific morphological characters within the genus during the last decade, 18S rRNA gene sequences were widely used for species identification. As coinfection patterns were recently reported from nuclear molecular data for two sympatric freshwater turtles *Mauremys leprosa* and *Emys orbicularis* from Tunisia, our objectives were to design COI specific primers to confirm the presence of three distinct species in both host species. Blood samples were collected from 22 turtles, from which DNAs were extracted and used as templates for amplification. Following different rounds of PCR and nested PCR, we designed specific *Haemogregarina* COI primers that allowed the sequencing of nine distinct haplotypes. Phylogenetic Bayesian analysis revealed the occurrence of three well-differentiated sublineages that clustered together into a single clade. Based on pairwise genetic distances (p-distance), we confirmed the occurrence of three distinct but phylogenetically closely related species coinfecting *M. leprosa* and *E. orbicularis* in the same aquatic environments. Our results demonstrate that the use of fast evolving genes within *Haemogregarina* will help to investigate the parasite diversity within both intermediate vertebrate and definitive invertebrate hosts, and to assess the evolution, historical biogeography and specificity of haemogregarines.

Keywords.

Haemogregarina - Freshwater turtles - *Mauremys leprosa* - *Emys orbicularis* - COI

Highlights.

- Species of *Haemogregarina* are blood parasites infecting, among others, freshwater turtles
- COI specific primers were designed to specifically amplify *Haemogregarina* spp.
- Sequencing revealed distinct parasite haplotypes within *M. leprosa* and *E. orbicularis*
- COI will help to investigate the parasite diversity within turtles and leeches

1. Introduction

The phylum Apicomplexa is a major group of protistan parasites that infect a wide variety of animals. While human pathogens, such as *Toxoplasma gondii*, *Babesia* spp., *Cryptosporidium* spp. and *Plasmodium* spp., have been extensively studied for their biological and genetic characteristics [1], the majority of apicomplexan parasites without medical importance are still poorly studied [2], yet they play a critical role in ecosystem functioning [3,4]. The identification and the description for all of these parasites, irrespective their degree of pathogenicity, remains a crucial step to encourage further studies in basic and applied research.

It is well known that morphological approaches alone can in some cases be misleading or unreliable in species identification due to the existence of cryptic taxa, differences in life stages or gender, genetic variability and/or phenotypic plasticity [5-7]. The DNA barcoding initiative that emerged following the development of molecular approaches in the 1990s now represents a central approach to circumvent taxonomic uncertainties [5]. Today, the cytochrome c oxidase I (COI) is still the most widely used marker for molecular identification at species level within taxa as diverse as arthropods, fish and birds [8-12]. Its maternal inheritance, limited recombination, robustness of mtDNA against degradation and rapid evolution make it the ideal marker for resolving recent phylogenetic events [13]. In contrast, the slow evolving nuclear 18S rRNA gene is one of the most valuable markers for resolving deep phylogenetic relationships [14-19].

Within the phylum of Apicomplexa, the most widely used mitochondrial markers for taxonomy, phylogeny and molecular idenification involved COI [20-22] and cytochrome b [2,23,24] sequences. Ogedengbe et al. [20] revealed that partial COI sequences provided many more variable characters than longer 18S rRNA gene sequences to distinguish closely related coccidian parasite species. On 07 January 2021, 308,627 COI sequences for

vertebrates were publically available from the National Center for Biotechnology Information (NCBI) compared to 10,307 18S sequences for the same group of species (keywords used: Vertebrata and COI *versus* Vertebrata and 18S). Conversely, only 1,303 COI sequences were found within the Apicomplexa from the NCBI compared to 20,903 18S sequences within the same phylum (keywords used: Apicomplexa and COI *versus* Apicomplexa and 18S), which showed that the 18S rRNA gene was still the most investigated molecular marker for phylogenetic studies of apicomplexan parasites. Such differences in the proportion of COI *versus* 18S sequences in these groups of taxa (5,8% of COI sequences for the Apicomplexa compared to 96,7% for the Vertebrata) could be partially explained by the difficulties associated with the design of universal and specific amplification primers in the COI for groups that are still poorly studied. Until now, there have only been few PCR primer sets used to amplify COI sequences within apicomplexan parasites. The universal primers LCO1490F and HCO2198R, which were proven to amplify a 658bp fragment of the COI gene in a wide range of invertebrates [25,26], were inadequate in the amplification process of the COI locus from protists and more specifically from apicomplexan parasites [27]. On the other hand, the high number of 18S copies and conserved terminal regions in the 18S rRNA locus made it easier to design such primers [28-30].

Haemogregarina is a genus which infects vertebrates as fish and freshwater turtles. It is classified in the family Haemogregarinidae of the order Eucoccidiorida. Until the 1990s, identification and recognition of *Haemogregarina* spp. were primarily based on morphological features. Due to limited interspecific morphological differences, traditional approaches were subsequently combined with molecular methods using 18S rRNA gene sequences for species identification [31]. Genetic diversity of blood parasites within the sympatric freshwater turtles *Mauremys leprosa* and *Emys orbicularis* of Tunisia, based on 18S sequences, revealed the occurrence of three distinct *Haemogregarina* variants including

H. stepanowi [32]. As coinfection patterns were clearly reported from molecular data for both turtle species, morphological identification of these variants was challenging, if not impossible [32]. Thus, our objectives in the present study were to design COI specific primers to investigate the COI *Haemogregarina* variations from blood samples in both turtle species and to then validate the presence of three distinct species of *Haemogregarina*.

2. Material and methods

2.1. Biological samples

Two freshwater turtle species, namely *M. leprosa*, the Mediterranean pond turtle, and *E. orbicularis*, the European pond turtle, were trapped using hoop nets baited with fish from three localities in Northern Tunisia (see Table 1 for GPS coordinates and also ref [32]). *Mauremys leprosa* was detected at all sampling sites corresponding to El Amaria, El Hania and Large Hammam Bourguiba pond, while *E. orbicularis* was found only at El Hania. Leeches were present on their vertebrate hosts in all localities. Blood samples were collected from the dorsal coccygeal sinus at the base of the turtle tail and preserved at -80°C in 98% ethanol until DNA extraction. A drop from each blood sample was also smeared on a glass slide, air-dried, fixed in absolute methanol for 5 min and stained with 10% Giemsa for 20 min in the laboratory for the presence of hemoparasites. Results from microscopic observations were reported in [32].

2.2. Primer design, DNA extractions, amplifications and sequencing

The first step involved the design of degenerate primers from an alignment comprising ten COI sequences of apicomplexan parasite species followed by the evaluation of their efficiency in amplifying *Haemogregarina* COI. These sequences, which were extracted from GenBank (see Table 2), corresponded to species belonging to Eucoccidiorida, Haemosporida

and Piroplasmida orders. They were aligned using Clustal [33] implemented in MEGA X software [34]. The most conserved regions in the 5' and 3' ends were selected to design three forward (HemFor1, HemFor2 and HemFor3) and three reverse (HemRev1, HemRev2 and HemRev3) degenerated primers (Table 3).

DNA was extracted from 22 blood samples of *M. leprosa* and *E. orbicularis* with the E.Z.N.A Tissue DNA Kit (Omega bio-tek, USA), according to manufacturer recommendations. Six DNA samples, four from *M. leprosa* (Ml36AM, Ml34AM, Ml44HS and Ml10HB) and two from *E. orbicularis* (Eo18HS and Eo19HS) were selected to test the PCR efficiency of the newly designed degenerated primers used two by two. These DNA samples were also studied earlier in [32] to investigate the genetic diversity of *Haemogregarina* based on 18S rRNA gene sequences. All PCRs were performed in a final volume of 25 µL under the following conditions: 5 min at 95°C for initial denaturation; 35 cycles of 30 sec each at 94°C for denaturation, 30 sec at 48°C for annealing, 1 min at 72°C for elongation; 10 min at 72°C for final extension. PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization.

Since all of the nine primer combinations failed to amplify the expected PCR fragments (Figure 1a), nested PCRs were subsequently performed from PCR products resulting from the amplification of Ml30AM and Eo19HS DNA samples with primers HemoFor1 and HemoRev1. These two new templates allowed for four combinations of inner primers to be tested, namely HemoFor2/HemoRev2, HemoFor2/HemoRev3, HemoFor3/HemoRev2 and HemoFor3/HemoRev3. Nested PCRs were conducted in a final volume of 25 µL following the same thermal conditions as described above. Only the HemoFor2/HemoRev2 primer combination allowed for the amplification of a PCR product of the expected size from the preamplified Ml30AM DNA sample (results not shown). Following sequencing by the Genoscreen Company (Lille, France), the chromatogram was

imported and checked with Chromas 2.2.6 (Technelysium Pty Ltd). Blast search of this fragment at 436bp revealed about 70% identity with COI sequences of apicomplexan parasites. As the occurrence of *Haemogregarina* parasites was evidenced from the same blood sample using morphological and molecular (18S rRNA gene sequences) approaches [32], the COI sequence we obtained was assigned to a species of *Haemogregarina*.

The resultant COI sequence was aligned with the ten selected apicomplexan COI sequences (see above) and used to develop six new specific PCR primers in the most conserved regions of the 5' and 3' ends. Two forward primers, namely HemoFor4 and HemoFor5, and four reverse primers, namely HemoRev4, HemoRev5, HemoRev6 and HemoRev7, were designed (Table 3). Subsequently all primers used two by two were tested on the six DNA samples that were used during the first step of amplification. Following the same thermal PCR conditions, with the exception of the annealing temperature that was fixed to 60°C, only four combinations of primers (HemoFor4/HemoRev4, HemoFor5/HemoRev4, HemoFor5/HemoRev6 and HemoFor5/HemoRev7) gave PCR fragments of the expected size with low PCR background for all six DNA samples (Figure 1b). These primers were thus used two by two to amplify the 22 DNA samples extracted from blood of *M. leprosa* and *E. orbicularis*. When the amplification step was successful with a strong PCR signal, PCR products were sent to the Genoscreen Company (Lille, France) for sequencing. Chromatograms were then imported and edited with Chromas 2.2.6 (Technelysium Pty Ltd) and resulting sequences were aligned using Clustal W implemented in MEGA X software.

2.3. Phylogenetic and distance analyses

Fifty-three COI sequences corresponding to species of distinct genera spanning Eucoccidiorida, Haemosporida and Piroplasmida orders were extracted from GenBank (Table 2) and aligned with the COI haplotypes confirmed within this study with Clustal W

implemented in MEGA X. Another COI sequence corresponding to *Scrippsiella sweeneyae* (Dinoflagellata) was also revovered from GenBank for outgroup comparison. A JC + G model was selected following the Akaike Information Criterion (AIC) implemented in Modeltest 3.06 [35]. A single substitution model and gamma rates with four categories were applied to the COI partition that comprised 418 characters. The Bayesian analysis was run using MrBayes 3.04b [36], with four chains running for five million generations and sampled every 100 cycles. The Bayesian consensus tree was drawn after removing the first 5,000 trees (10%) as the burn-in phase and opened under TreeView version 1.6 [37]. Pairwise distances (p-distance) were also estimated both within two closely related species of *Eimeria* (*E. lancasterensis* and *E. sciurorum*) as well as between these two species, to assess levels of genetic divergences for species delimitation within the Eucoccidiorida.

3. Results

Following amplification of the 22 DNA samples using the four specific primer combinations, 21 were positive using HemoFor4/HemoRev4 primer set of which 11 were sequenced, 21 were positive using the HemoFor5/HemoRev4 primer set of which 14 were sequenced, 22 were positive using the HemoFor5/HemoRev6 primer set of which 11 were sequenced and 21 were positive using the HemoFor5/HemoRev7 primer set of which 18 were sequenced. Between one and three distinct haplotypes were identified per blood sample depending on the combination of primers used for PCR, indicating that several genetic variants may occur in the same turtle specimen. A total of nine distinct COI haplotypes were identified (Table 4).

The Bayesian analysis of the COI sequences resulted in a tree which grouped all of the COI *Hamogregarina* haplotypes together in a single clade, which can be subdivided into three distinct sublineages: *Haemogregarina* sp_Hap1 includes haplotypes 1a and 1b, *Haemogregarina* sp_Hap2 includes a single haplotype 2a and *Haemogregarina* sp_Hap3

includes haplotypes 3a to 3f. Isolates of the species of *Haemogregarina* were most closely related to *Hepatozoon* as compared to any of the other apicomplexans (Figure 2). While pairwise distances within both *Haemogregarina* sublineages Hap1 and Hap 3 were less than 0,01 on average (Table 5), they were higher between the three distinct sublineages (Table 6). Pairwise distances were 0.077 between *Haemogregarina* sp_Hap1 and *Haemogregarina* sp_Hap2, ranged from 0,066 to 0,074 with an average of 0,069 ± 0,003 between *Haemogregarina* sp_Hap1 and *Haemogregarina* sp_Hap3 and ranged from 0,086 to 0,094 with an average of 0,089 ± 0,003 between *Haemogregarina* sp_Hap2 and *Haemogregarina* sp_Hap3 (Table 6). For comparison, intra specific pairwise distances within *Eimeria lancasterensis* and *E. sciurorum* were less than 0,006 on average (Table 5), and interspecific pairwise distances between these two species ranged from 0,014 to 0,029 with an average of 0,021 ± 0,005 (Table 6).

4. Discussion

Although the mitochondrial COI is the most widely used marker for DNA barcoding across most animal taxa [8-12], the use of COI sequences as DNA barcodes within Protozoa is still in its initial stage. While the usefulness of this genetic marker for differentiation of cryptic species has already been proven successful for several apicomplexans [38-41], no species of the Haemogregarinidae was processed until now for COI barcodes [42]. In addition, molecular species identification of *Haemogregarina* was solely based on 18S rRNA gene sequences, which may hidden the true species diversity. The molecular characterization of *Haemogregarina* using specific COI primers revealed the presence of nine distinct haplotypes within the two freshwater turtles *E. orbicularis* and *M. leprosa* in Tunisia. Based on Bayesian analyses, *Haemogregarina* COI haplotypes formed a monophyletic group that was subdivided into three sub-lineages, namely *Haemogregarina* sp_Hap1, *Haemogregarina* sp_Hap2 and

H*aemogregarina* sp_Hap3 (Figure 2). Pairwise distances estimated between the three sub-lineages are greater than 6.6%, which is much higher than the interspecific average pairwise distance, i.e. 2.1%, estimated between two valid distinct species, namely *E. lancasterensis* and *E. sciurorum* (Table 6). Additionally, the pairwise distances within *Haemogregarina* sp_Hap1 and within *Haemogregarina* sp_Hap3 are less than 1.1% (Table 5). These outcomes suggest that each *Haemogregarina* sub-lineage may represent a distinct species, which was previously suggested by Attia El Hili et al. [32] based on nuclear 18S rRNA gene sequences. Furthermore the current study showed that the COI gene is a more variable marker than the 18S gene indicating that the COI gene may be useful in both systematics as well as in phylogeography studies of species of *Haemogregarina*. For all these reasons, and because it is not necessary to sequence the total length of the COI to get variable and informative characters, focusing on COI instead of 18S marker may be more advantageous in terms of cost and time.

The occurrence of several COI variants within some turtle specimens is in agreement with the molecular results inferred from the analysis of 18S rRNA gene sequences obtained from the same blood samples [32]. This confirms co-infection patterns of species of *Haemogregarina* within freshwater turtles in Tunisia, which was also observed in other apicomplexans such as species of *Hepatozoon*, *Eimeria* and *Isospora* of Eucoccidiorida [43-46], and species of *Plasmodium* and *Haemoproteus* of Haemosporida [47]. Due to the presence of several *Haemogregarina* COI variants in the same turtle specimen, as is also the case with *Haemogregarina* 18S variants [32], it remains difficult to associate a particular COI haplotype to one of the three identified 18S haplotypes. Besides *H. stepanowi*, for which it is still impossible to assign a particular COI haploclade, the morphological description of the two new *Haemogregarina* species will be possible once unique COI and 18S haplotypes are identified from the same turtle population.

Based on morphological and molecular analyses, a low diversity of *Haemogregarina* species, accounting for less than 20 species [31,32,48-54], successfully infect freshwater turtles. With the exception of Attia El Hili et al. [32] who reported from 18S rRNA gene sequences the presence of three *Haemogregarina* species within the same individuals, all other reports that focused on both morphological and genetic data of *Haemogregarina* revealed the presence of single or low divergent 18S variants per individual, suggesting the occurrence of a single parasite species in sympatric hosts [31,48-54]. Following PCR assays that were conducted on haemosporidian parasites, Bernotienė et al. [47] showed several cases of mixed infections indicating that single PCR assays could underestimate the true parasitic diversity. This can explain why, until recently [32], so few *Haemogregarina* species were documented within wild freshwater turtles from 18S rRNA gene sequences. Because of the low specificity of these parasites towards their chelonian hosts, the use of fast evolving mitochondrial markers should help to assess the true diversity of *Haemogregarina* infections within turtles. Similarly, investigating this parasite diversity within leeches may provide new insights into the specificity of species of *Haemogregarina* and their definitive hosts. Finally, even if *M. leprosa* is found predominantly in the Southern parts of the Mediterranean basin, and *E. orbicularis* in the Northern parts [55], they can also be found in sympatry in some areas of Spain and Portugal, as well as in restricted areas of Southern France, Morocco, Algeria and Tunisia. Therefore, it may be interesting to investigate the genetic diversity of *Haemogregarina* spp. in both host species along their range in order to focus on the overall parasite diversity and specificity and to further explore additional aspects of their origin, evolution and historical biogeography.

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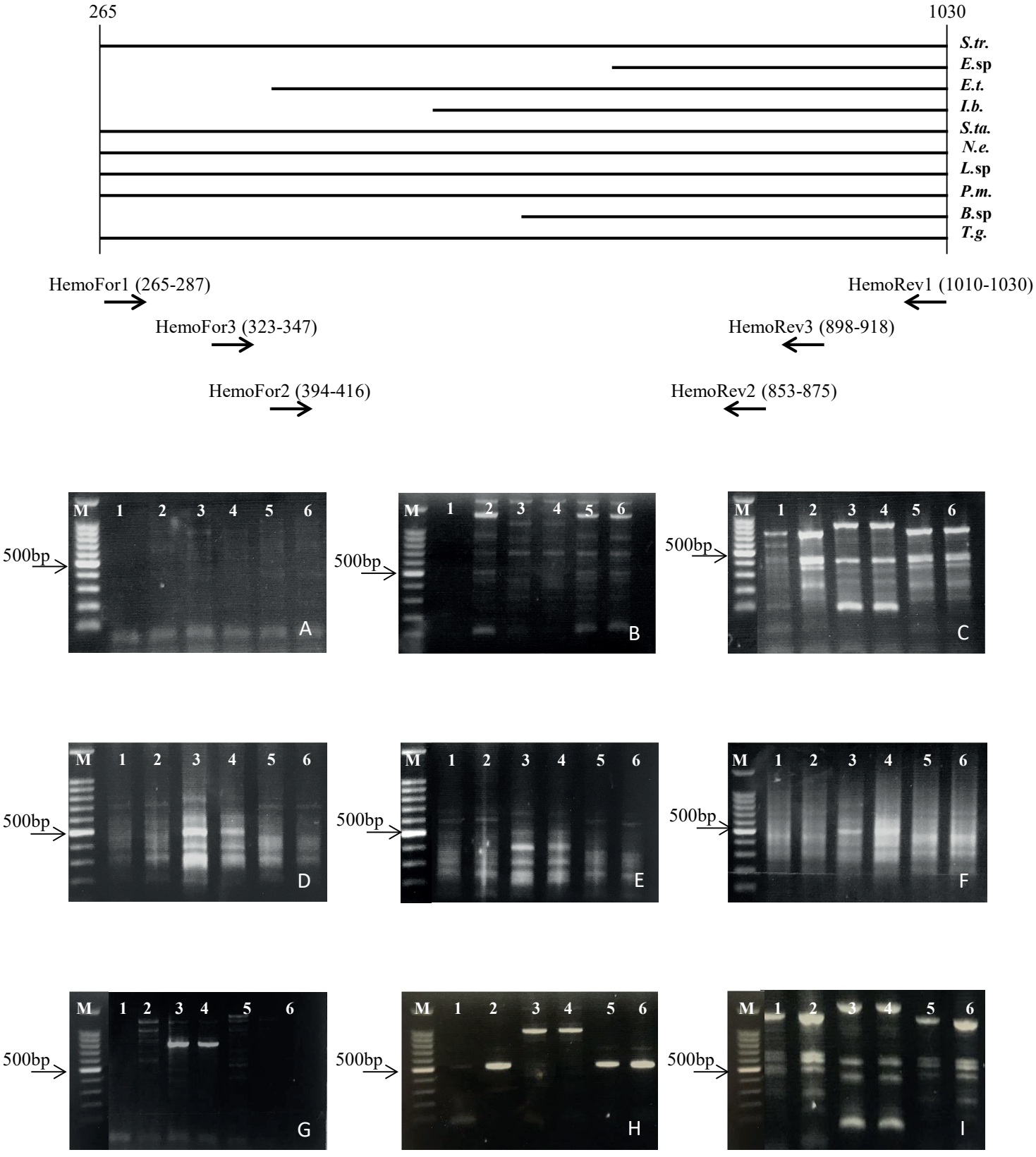
Figure legends

Figure 1a. Schematized alignment of apicomplexan COI sequences, from top to bottom *Sarcocystis truncate* (S.tr.), *Eimeria* sp (E.sp), *Eimeria tenella* (E.t.), *Isospora butcherae* (I.b.), *Sarcocystis tarandi* (S.ta.), *Nephroisospora eptesici* (N.e.), *Leucocytozoon* sp (L.sp), *Plasmodium malariae* (P.m.), *Babesia* sp (B.sp) and *Toxoplasma gondii* (T.g.), used to design degenerated primers HemoFor1, HemoFor2, HemoFor3, HemoRev1, HemoRev2 and HemoRev3 for PCR (not at scale). Numbers in brackets refer to primers position after mapping on the longest COI sequence extracted from GenBank, namely *T. gondii* COI. Photographs A to I show PCR products resulting from amplification of DNAs Ml36AM (well n°1), Ml34AM (well n°2), Ml44HS (well n°3), Ml10HB (well n°4), Eo18HS (well n°5) and Eo19HS (well n°6) with HemoFor1 - HemoRev1 in A (expected size ≃ 765bp), HemoFor1 - HemoRev2 in B (expected size ≃ 610bp), HemoFor1 - HemoRev3 in C (expected size ≃ 655bp), HemoFor2 – HemoRev1 in D (expected size ≃ 635bp), HemoFor2 - HemoRev2 in E (expected size ≃ 480bp), HemoFor2 - HemoRev3 in F (expected size ≃ 525pb), HemoFor3 - HemoRev1 in G (expected size ≃ 705pb), HemoFor3 - HemoRev2 in H (expected size ≃ 550pb) and HemoFor3 - HemoRev3 in I (expected size ≃ 595pb).

Figure 1b. Schematized alignment of apicomplexan COI sequences, from top to bottom *Sarcocystis truncate* (S.tr.), *Eimeria* sp (E.sp), *Eimeria tenella* (E.t.), *Isospora butcherae* (I.b.), *Sarcocystis tarandi* (S.ta.), *Nephroisospora eptesici* (N.e.), *Leucocytozoon* sp (L.sp), *Plasmodium malariae* (P.m.), *Babesia* sp (B.sp), *Toxoplasma gondii* (T.g.) and *Mauremys leprosa* (M.l.30AM), used to design primers HemoFor4, HemoFor5, HemoRev4, HemoRev6 and HemoRev7 for PCR (not at scale). Numbers in brackets refer to primers position after mapping on the longest COI sequence extracted from GenBank, namely *T. gondii* COI.

Photographs J to M show PCR products resulting from amplification of DNAs Ml36AM (well n°1), Ml34AM (well n°2), Ml44HS (well n°3), Ml10HB (well n°4), Eo18HS (well n°5) and Eo19HS (well n°6) with HemoFor4 – HemoRev4 in J (expected size ≃ 465bp), HemoFor5 – HemoRev4 in K (expected size ≃ 410bp), HemoFor5 – HemoRev6 in L (expected size ≃ 400bp) and HemoFor5 – HemoRev7 in M (expected size ≃ 405bp).

Figure 2. Bayesian tree. Values next to nodes refer to Bayesian posterior probabilities and numbers in brackets refer to Genbank Accession numbers for sequences extracted from GenBank as well as for new sequences.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HemoRev1 (1010-1030)
HemoFor3 (323-347)
HemoRev3 (898-918)
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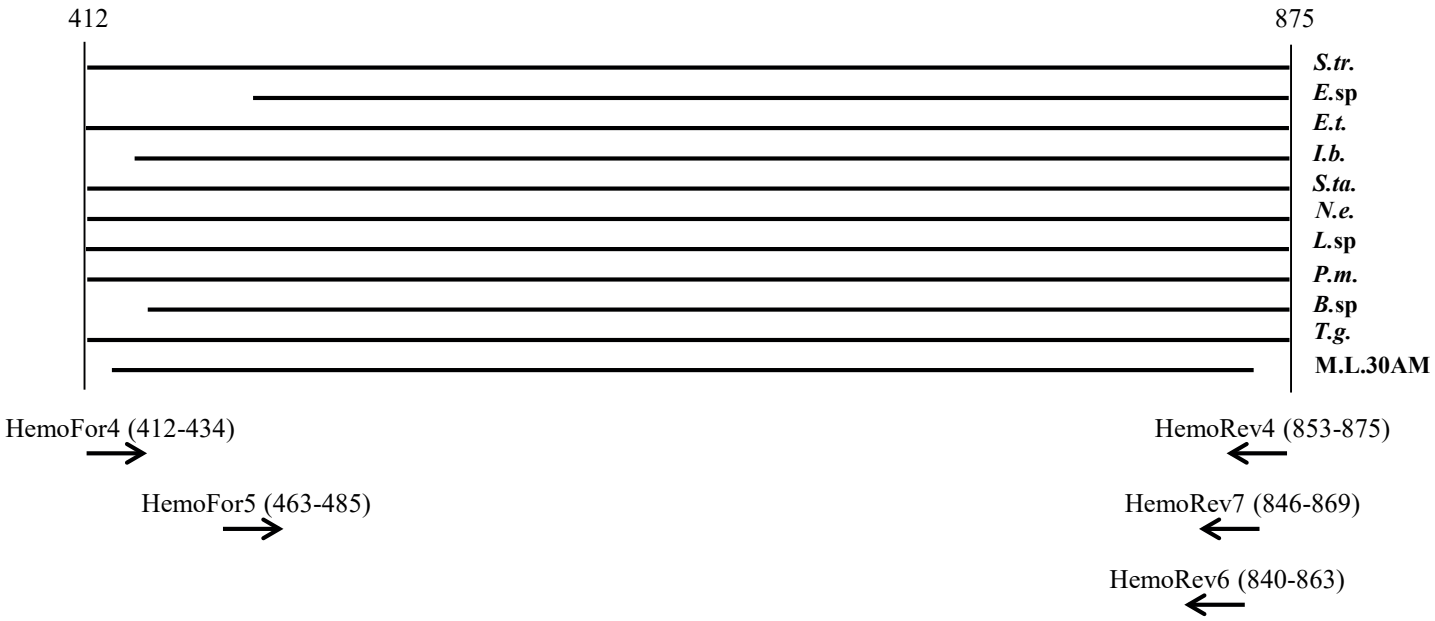
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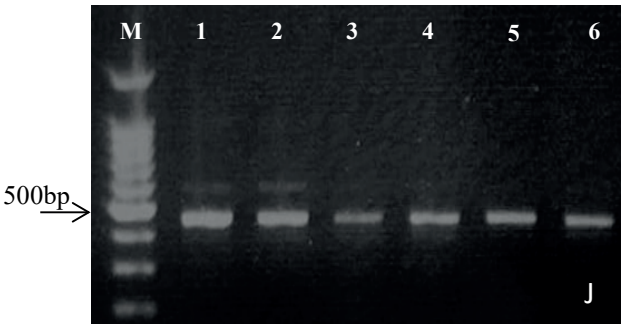
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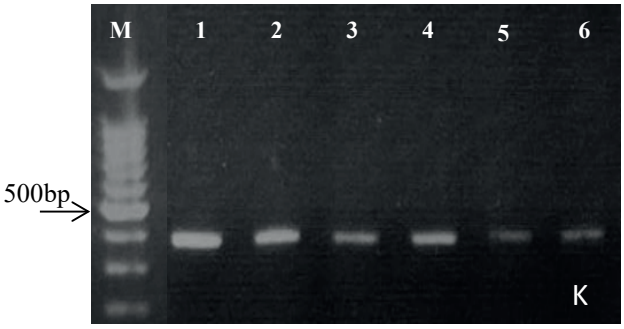
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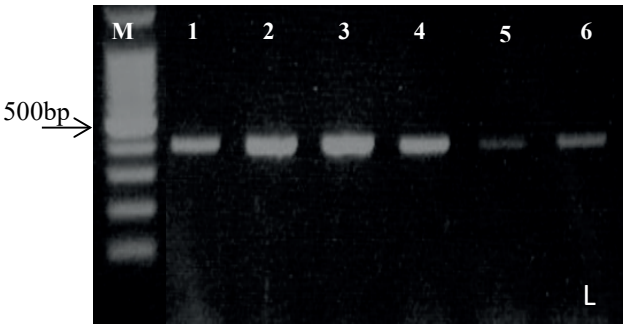
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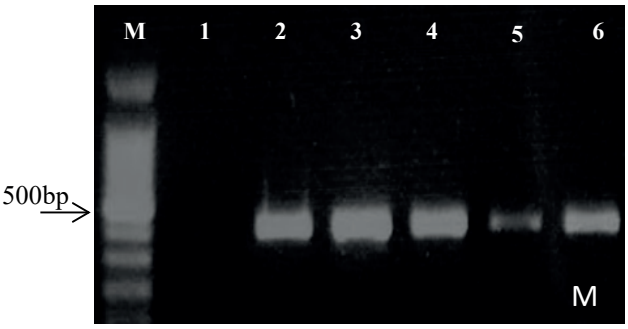
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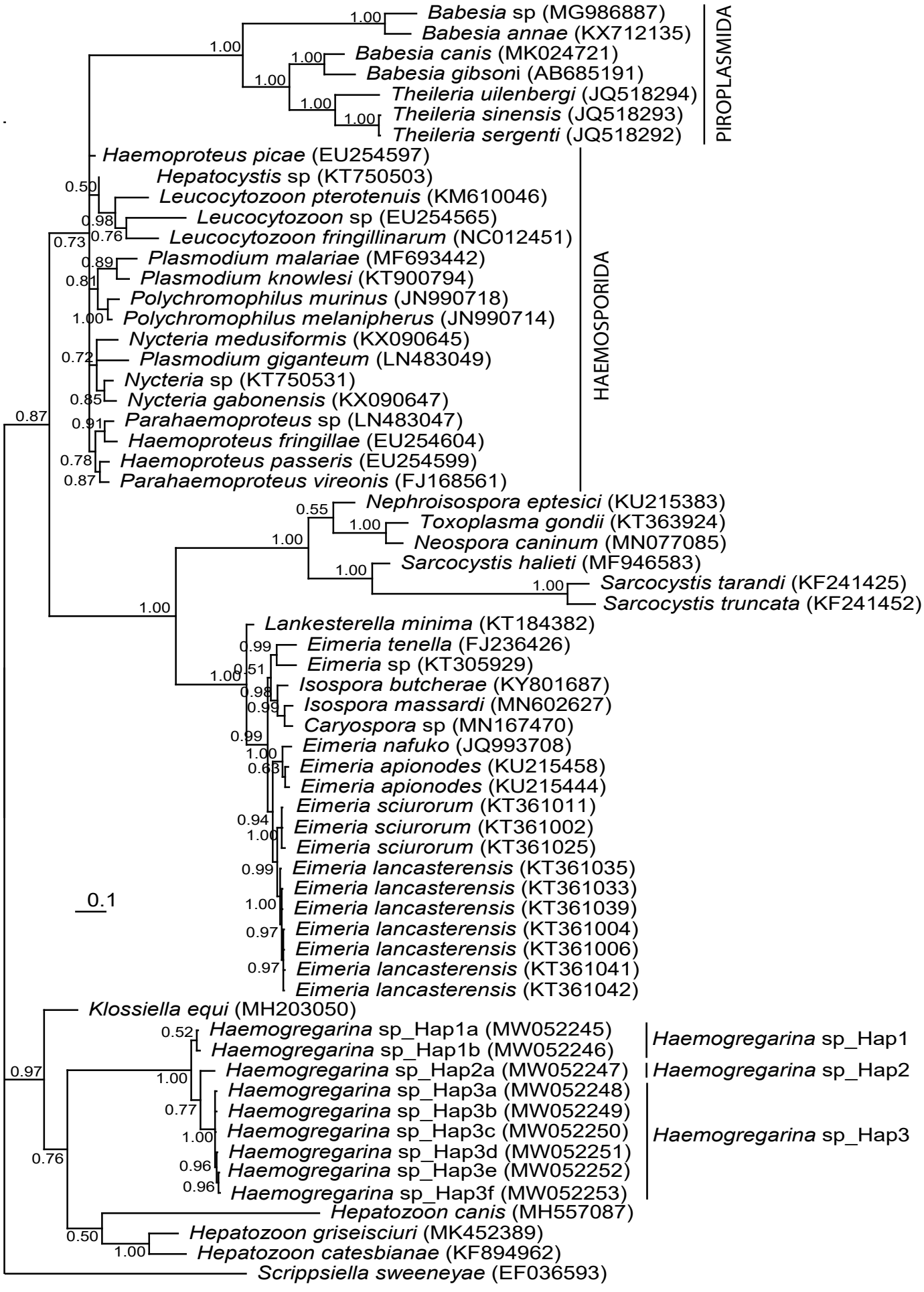
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Theileria uilenbergi (JQ518294)
Theileria sinensis (JQ518293)
Theileria sergenti (JQ518292)
PIROPLASMIDA
Haemoproteus picae (EU254597)
Hepatocystis sp (KT750503)
Leucocytozoon pterotenuis (KM610046)
Leucocytozoon sp (EU254565)
Leucocytozoon fringillinarum (NC012451)
Plasmodium malariae (MF693442)
Plasmodium knowlesi (KT900794)
Polychromophilus murinus (JN990718)
Polychromophilus melanipherus (JN990714)
Nycteria medusiformis (KX090645)
Plasmodium giganteum (LN483049)
Nycteria sp (KT750531)
Nycteria gabonensis (KX090647)
Parahaemoproteus sp (LN483047)
Haemoproteus fringillae (EU254604)
Haemoproteus passeris (EU254599)
Parahaemoproteus vireonis (FJ168561)
HAEMOSPORIDA
Nephroisospora eptesici (KU215383)
Toxoplasma gondii (KT363924)
Neospora caninum (MN077085)
Sarcocystis halieti (MF946583)
Sarcocystis tarandi (KF241425)
Sarcocystis truncata (KF241452)
Lankesterella minima (KT184382)
Eimeria tenella (FJ236426)
Eimeria sp (KT305929)
Isospora butcherae (KY801687)
Isospora massardi (MN602627)
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Klossiella equi (MH203050)
Haemogregarina sp_Hap1a (MW052245)
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Haemogregarina sp_Hap2a (MW052247)
Haemogregarina sp_Hap2
Haemogregarina sp_Hap3a (MW052248)
Haemogregarina sp_Hap3b (MW052249)
Haemogregarina sp_Hap3c (MW052250)
Haemogregarina sp_Hap3d (MW052251)
Haemogregarina sp_Hap3e (MW052252)
Haemogregarina sp_Hap3f (MW052253)
Haemogregarina sp_Hap3
Hepatozoon canis (MH557087)
Hepatozoon griseisciuri (MK452389)
Hepatozoon catesbianae (KF894962)
Scrippsiella sweeneyae (EF036593)

Table 1. GPS coordinates of sampling sites

Locality	Latitude	Longitude
El Amaria	37.19667	9.40194
El Hania	37.11059	9.21056
Large Hammam Bourguiba pond	36.7625	8.55167

Table 2. GenBank accession numbers for sequences used in phylogenetic analyses

Parasite species	Host species	Geographical origin	Accession No	Reference
Eucoccidiorida				
Caryospora sp.	*Grallina cyanoleuca*	Australia	MN167470	[56]
Eimeria apionodes	rodent	Bulgaria	KU215444	[57]
Eimeria apionodes	rodent	Bulgaria	KU215458	[57]
Eimeria lancasterensis	*Sciurus carolinensis*	Italy	KT361004	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361006	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361033	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361035	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361039	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361041	[58]
Eimeria lancasterensis	*S. carolinensis*	Italy	KT361042	[58]
Eimeria nafuko	*Heliophobius argenteocinereus*	Czech Republic	JQ993708	[59]

Eimeria sciurorum	*Sciurus vulgaris*	Italy	KT361002	[58]
Eimeria sciurorum	*S. vulgaris*	Italy	KT361011	[58]
Eimeria sciurorum	*S. vulgaris*	Italy	KT361025	[58]
*Eimeria tenella**	chicken	USA	FJ236426	[60]
Eimeria sp*	*Columba livia domestica*	Australia	KT305929	[61]
Haemogregarina sp_Hap1a	*M. leprosa / E. orbicularis*	Tunisia	MW052245	This study
Haemogregarina sp_Hap1b	*M. leprosa / E. orbicularis*	Tunisia	MW052246	This study
Haemogregarina sp_Hap2a	*M. leprosa / E. orbicularis*	Tunisia	MW052247	This study
Haemogregarina sp_Hap3a	*M. leprosa / E. orbicularis*	Tunisia	MW052248	This study
Haemogregarina sp_Hap3b	*M. leprosa / E. orbicularis*	Tunisia	MW052249	This study
Haemogregarina sp_Hap3c	*M. leprosa / E. orbicularis*	Tunisia	MW052250	This study
Haemogregarina sp_Hap3d	*M. leprosa / E. orbicularis*	Tunisia	MW052251	This study
Haemogregarina sp_Hap3e	*M. leprosa / E. orbicularis*	Tunisia	MW052252	This study
Haemogregarina sp_Hap3f	*M. leprosa / E. orbicularis*	Tunisia	MW052253	This study
Hepatozoon canis	*Canis lupus familiaris*	Israel	MH557087	[42]
Hepatozoon catesbianae	*Lithobates clamitans*	USA	KF894962	[62]

Hepatozoon griseisciuri	*Sciurus carolinensis*	Canada	MK452389	[63]
Klossiella equi	*Equus ferus caballus*	USA	MH203050	[64]
*Isospora butcherae**	*Zosterops lateralis*	Australia	KY801687	[65]
Isospora massardi	*Turdus flavipes*	Brazil	MN602627	[66]
Lankesterella minima	*Lithobates clamitans*	Canada	KT184382	[67]
Neospora caninum	-	-	MN077085	[68]
*Nephroisospora eptesici**	*Eptesicus fuscus*	USA	KU215383	[67]
Sarcocystis halieti	*Haliaeetus albicilla*	Norway	MF946583	[69]
*Sarcocystis truncata**	*Cervus elaphus*	Norway	KF241452	[38]
*Sarcocystis tarandi**	*Rangifer tarandus*	Norway	KF241425	[38]
*Toxoplasma gondii**	-	-	KT363924	[27]
Haemosporida				
Haemoproteus fringillae	*Zonotrichia albicollis*	USA	EU254604	[70]
Haemoproteus passeris	*Passer moabiticus*	Israel	EU254599	[70]
Haemoproteus picae	*Picoides pubescens*	USA	EU254597	[70]
Hepatocystis sp	*Epomops franqueti*	Uganda	KT750503	[71]

Leucocytozoon fringillinarum	*Pipilo chlorurus*	-	NC012451	[72]
Leucocytozoon pterotenuis	*Grallaria ruficapilla*	Colombia	KM610046	[73]
Leucocytozoon sp*	*Buteo lineatus*	USA	EU254565	[70]
Nycteria gabonensis	*Rhinolophus alcyone*	Congo	KX090647	[2]
Nycteria medusiformis	*Nycteris grandis*	Congo	KX090645	[2]
Nycteria sp	*Hipposideros cyclops*	Uganda	KT750531	[71]
Parahaemoproteus vireonis	*Vireo gilvus*	-	FJ168561	[72]
Parahaemoproteus sp	*Acrocephalus scirpaceus*	Germany	LN483047	[74]
Plasmodium giganteum	*Agama agama*	Nigeria	LN483049	[74]
Plasmodium knowlesi	*Macaca fascicularis*	Malaysia	KT900794	[75]
*Plasmodium malariae**	*Homo sapiens*	Cameroon	MF693442	[76]
Polychromophilus melanipherus	*Miniopterus schreibersii*	Switzerland	JN990714	[77]
Polychromophilus murinus	*Myotis daubentonii*	Switzerland	JN990718	[77]
Piroplasmida				
Babesia annae	*Canis aureus*	Romania	KX712135	[78]
Babesia canis	*Canis lupus familiaris*	Poland	MK024721	[79]

Babesia gibsoni	*Canis familiaris*	Japan	AB685191	[80]
Babesia sp*	*Procyon lotor*	USA	MG986887	[81]
Theileria sinensis	cattle	China	JQ518293	[82]
Theileria sergenti	cattle	China	JQ518292	[82]
Theileria uilenbergi	sheep	China	JQ518294	[82]
Outgroup (Dinoflagellata)				
Scrippsiella sweeneyae	-	-	EF036593	[83]

- : Not available; *: Sequences used to design degenerated primers

Table 3. List of PCR primers designed along this study

Primers	Sequences
HemoFor1	5'- GGATWTGGTAAYTWCTTYSTACC-3'
HemoFor2	5'- TTCGGTRSTGGYATKGGYTGGAC-3'
HemoFor3	5'- GAATTAACGCMRTWTCYTACTTCCT-3'
HemoFor4	5'-TGGACATTATACCCACCTTTAAG-3'
HemoFor5	5'- GCCGTAGACGCTATAATCTTAGG -3'
HemoRev1	5'- AWTKASTWSCCATATAAGTAC-3'
HemoRev2	5'- ATACAWCCCATRCGTAARATCAT-3'
HemoRev3	5'- TGTCATCATATGRTGTGCCCA-3'
HemoRev4	5'-ATACAACCCATAGCTAGTATCAT-3'
HemoRev5	5'-TTATTGTAGGAAGCGGATATCAC-3'
HemoRev6	5'-GCTAGTATCATAGCGTGGTTTCCG-3'
HemoRev7	5'-CCCATAGCTAGTATCATAGCGTGG-3'

Table 4. COI Haplotype diversity of *Haemogregarina* depending of the specific combination of primers used for PCR

Host species	Investigated specimen	HemoFor4/HemoRev4	HemoFor5/HemoRev4	HemoFor5/HemoRev6	HemoFor5/HemoRev7
E. orbicularis	EO11HS	+	Haplotypes 3b / 3c	Haplotype 3b	-
E. orbicularis	EO12HS	+	+	+	Haplotype 3e
E. orbicularis	EO18HS	Haplotypes 2a / 3b	Haplotype 3c	Haplotype 3c	Haplotype 3c
E. orbicularis	EO19HS	Haplotype 1b	Haplotype 3f	Haplotypes 3e / 3f	Haplotypes 3e / 3f
E. orbicularis	EO20HS	+	Haplotype 3c	Haplotype 3c	Haplotype 3c
M. leprosa	ML32HS	Haplotype 1a	Haplotype 3e	Haplotype 3e	Haplotype 3a
M. leprosa	ML43HS	+	-	+	+
M. leprosa	ML44HS	Haplotype 3d	Haplotypes 3b / 3d	Haplotype 3d	Haplotypes 3b / 3d

M. leprosa	ML45HS	+	+	+	+
M. leprosa	ML26AM	Haplotype 2a	Haplotype 3a	+	Haplotype 3a
M. leprosa	ML30AM	Haplotype 2a	Haplotype 3a	+	Haplotype 3a
M. leprosa	ML31AM	+	+	+	Haplotype 3a
M. leprosa	ML32AM	+	+	+	Haplotype 3a
M. leprosa	ML33AM	Haplotypes 2a / 3b	Haplotype 3c	Haplotype 3c	Haplotype 3b
M. leprosa	ML34AM	Haplotype 2a	Haplotype 3a	Haplotype 3a	Haplotype 3a
M. leprosa	ML35AM	+	+	+	Haplotype 3a
M. leprosa	ML36AM	Haplotype 2a	Haplotype 3a	Haplotype 3a	Haplotype 3a
M. leprosa	ML37AM	+	+	+	Haplotype 3a
M. leprosa	ML3HB	Haplotype 1b	Haplotype 1b	Haplotype 1b	+

M. leprosa	ML4HB	-	+	+	Haplotype 3e
M. leprosa	ML5HB	+	Haplotype 3e	+	Haplotype 3f
M. leprosa	ML10HB	Haplotype 1b	Haplotype 1b	Haplotype 3e	Haplotypes 1b / 3e

- : Absence of PCR amplification; + : PCR product not sequenced.

Table 5. Intraspecific pairwise distances

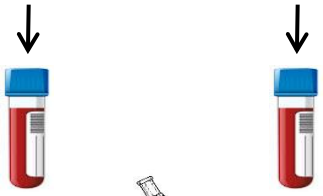
Parasite species	Minimum distance	Maximum distance	Average distance ± sd
Eimeria lancasterensis	0	0,006	0,002 ± 0,002
Eimeria sciurorum	0	0,009	0,006 ± 0,005
Haemogregarina sp_Hap1	0.006	0.006	NA
Haemogregarina sp_Hap3	0,003	0,011	0,006 ± 0,003

NA: Not applicable

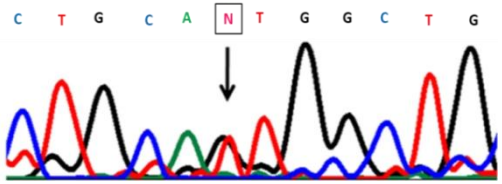
Table 6. Interspecific pairwise distances

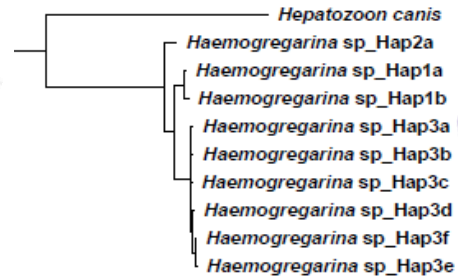
Species of comparison	Minimum distance	Maximum distance	Average distance ± sd
Eimeria lancasterensis - *Eimeria sciurorum*	0,014	0,029	0,021 ± 0,005
Haemogregarina sp_Hap1 - *Haemogregarina* sp_Hap2	0,077	0,077	0,077 ± 0
Haemogregarina sp_Hap1 - *Haemogregarina* sp_Hap3	0,066	0,074	0,069 ± 0,003
Haemogregarina sp_Hap2 - *Haemogregarina* sp_Hap3	0,086	0,094	0,089 ± 0,003

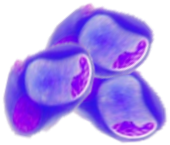
Mauremys leprosa *Emys orbicularis*

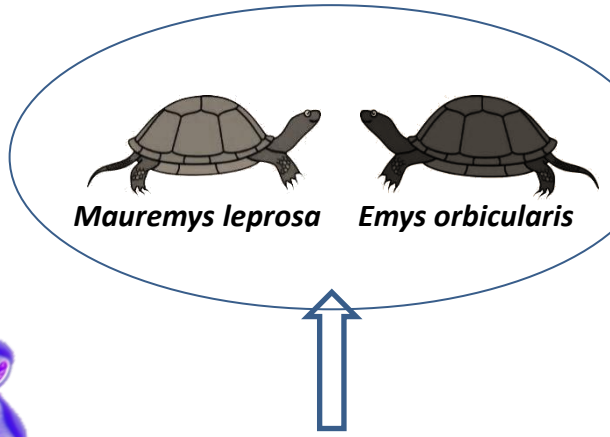


Heamogregarina









Three *Haemogregarina* species (sp_Hap1, sp_Hap2, sp_Hap3)