Parasitology International 65 (2016) 5–11

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Reconstruction of the evolutionary history of Haemosporida (Apicomplexa) based on the cyt *b* gene with characterization of *Haemocystidium* in geckos (Squamata: Gekkota) from Oman

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ARTICLE INFO

Article history:
Received 4 April 2015
Received in revised form 7 September 2015
Accepted 10 September 2015
Available online 12 September 2015

Keywords:
Haemoproteus
Plasmodium
Haemocystidium
Leucocytozoon
Vector association
Life-history traits
Bayesian Inference
Maximum Likelihood

ABSTRACT

The order Haemosporida (Apicomplexa) includes many medically important parasites. Knowledge on the diversity and distribution of Haemosporida has increased in recent years, but remains less known in reptiles and their taxonomy is still uncertain. Further, estimates of evolutionary relationships of this order tend to change when new genes, taxa, outgroups or alternative methodologies are used. We inferred an updated phylogeny for the Cytochrome *b* gene (cyt *b*) of Haemosporida and screened a total of 80 blood smears from 17 lizard species from Oman belonging to 11 genera. The inclusion of previously underrepresented genera resulted in an alternative estimate of phylogeny for Haemosporida based on the cyt *b* gene. *Leucocytozoon* and *Haemoproteus* appear as sister taxa to avian and reptilian *Plasmodium* based on Bayesian Inference (BI) analysis, in contrast with the most recent phylogenetic assessment of the evolutionary history of Haemosporida. Paraphyly of mammalian *Plasmodium* and polyphyly of avian *Haemoproteus* support the division into subgenera as suggested in other studies. Avian and Reptilian *Plasmodium* are also polyphyletic, and therefore a similar division may be needed. Reptilian *Haemocystidium* form a monophyletic lineage, sister taxa to avian and reptilian *Plasmodium* and mammalian *Polychromophilus* in the BI analysis but its position in the Maximum Likelihood (ML) analysis is not well supported. This supports its recent reclassification as a separate genus. Our results further corroborate the hypothesis that the phylogeny based on the cyt *b* gene is associated with the invertebrate host families that transmit each genus rather than parasite life-history traits. *Haemocystidium* sp. was detected in two geckos from Oman, which were related with *Haemocystidium* sp. from Malagasy *Oplurus* sp. (<2% genetic divergence) but diverged from other reptilian *Haemocystidium* species by more than 5%. It is likely that these represent a new *Haemocystidium* species and further research on the distribution of vectors and host-vector associations is needed.

1. Introduction

The apicomplexan order Haemosporida includes many medically important parasites, including *Plasmodium* spp. These parasites may be of major epidemiological concern and exert great economic costs to animal and public health, causing serious health issues including host death [1,2]. They are vector-borne, with sexual stages occurring in invertebrate hosts [3,4]. Apart from the threats posed to human health, these parasites are of concern in conservation because they can lead to extinction of naïve host species [5]. Despite the recent increase in the study of these parasites in some groups of wild hosts, particularly avian hosts [6,7], the distribution and diversity of these parasites in some hosts remain poorly investigated, especially in less studied host groups, such as reptiles from remote geographical regions.

Haemosporida includes four families: Garniidae, Haemoproteidae, Leucocytozoidae and Plasmodiidae that can be distinguished by life-history traits, such as the presence or absence of hemozoin pigmentation and the gain or loss of merogony [8,9]. However, most species within this order belong to three genera, *Plasmodium* (Plasmodiidae), *Leucocytozoon* (Leucocytozoidae) and *Haemoproteus* (Haemoproteidae) [10]. The study of Haemosporida has been primarily focused on the genus *Plasmodium* from a wide range of host groups, including primates [11,12], rodents [13,14], avian [15,16] and reptilian hosts [10,17], and more recently on avian *Haemoproteus* [18,19].

The evolutionary history of haemosporidians has long puzzled parasitologists with many hypotheses arising over time, often limited by the methodologies and analytical tools available [9]. Traditionally, the

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http://dx.doi.org/10.1016/j.parint.2015.09.003
1383-5769/

main characters for describing species and inferring their evolutionary history were based on the vertebrate host taxon and parasite traits observed through microscopy. However, these traits may have evolved convergently to respond to ecological changes, such as host switches [10], and the vertebrate host taxon may not be an adequate taxonomic character due to frequent vertebrate host switches among haemosporidians [20]. For this reason, the implementation of molecular tools to estimate the phylogenetic relationships between haemosporidians has shed new light into their evolutionary history. Initial molecular studies were based on few sequences and thus were prone to taxon sampling biases [21], inappropriate rooting [22] and gene limitations [23]. In fact, tree topologies vary greatly depending on the outgroup and rooting approach [9,24], and on the number of taxa and representatives of each genus [25–28]. Hence, it is important that large-scale phylogenetic reconstructions are conducted from time to time to re-assess the relationships between genera. Studies comparing the resolution of morphological characters, mitochondrial and nuclear genes in *Haemoproteus* species indicate that the diversity observed in the mitochondrial Cytochrome *b* (cyt *b*) gene is representative of the level of differentiation genome-wide [29,30]. For this reason, this gene is the most widely used for taxonomic purposes and to assess the evolutionary history of Haemosporida. The cut-off for identifying new species is considered above 5% in cyt *b*, although there are instances of species that differ by only 1% or less [18,31,32]. In addition, multi-gene phylogenies have provided important information, for example that the major cladogenic events of Haemosporida seemed to be related to switches between vector hosts [10].

Considerable effort has been put into investigating the diversity of haemosporidians in avian hosts, with many studies analyzing the prevalence, intensity and phylogenetic relationships of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* [33]. These studies have shown that the number of species initially estimated based on microscopy is an underestimation of the real diversity of this group of parasites [8,33]. On the other hand, studies on Haemosporida of reptiles are still scarce, with only a few descriptions of *Haemoproteus* species, such as *Haemoproteus mesnili* and *Haemoproteus balli* in snakes [34], and *Haemoproteus kopki* and *Haemoproteus ptyodactylii* in lizards [28]. A recent molecular study showed that the reptilian *Haemoproteus* clade is clearly distinct from avian *Haemoproteus* and thus should be reclassified as the genera *Haemocystidium* [35].

In this study, we examined the phylogenetic relationships of Haemosporida based on the mitochondrial cyt *b* gene, with a focus on parasites of reptiles, and conducted a morphological and molecular characterization of *Haemocystidium* from geckos from Oman. Information regarding distribution of parasites in these hosts is currently scarce, even though this region is known to have a high degree of reptile endemism and diversity (e.g. [36–38]).

2. Materials and methods

2.1. Microscopic examination

A total of 80 blood smears from geckos from Oman (collected in May 2011) (see Table A.1 for exact GPS coordinates) were screened for the presence of haemosporidian parasites (Table 1). Blood smears were air-dried, fixed with methanol and stained with diluted Giemsa (1:9 of distilled water) for 55 min. Blood smears were screened using an Olympus CX41 microscope with an in-built digital camera (SC30) (Olympus, Hamburg, Germany). Intensity of infection was estimated as the number of parasites per 4,000 erythrocytes (Table 1). Intracellular parasites and infected host erythrocytes were measured at 1000× magnification (Table 2) using cell ^B software (basic image acquisition and archiving software; Olympus, Münster, Germany). Length, width, vertical and horizontal distance were taken using polygon and arbitrary distance tools, while the area and perimeter were taken using the area/perimeter tool in the Measure menu of cell ^B software.

Table 1
Blood smear samples analysed for Haemosporida parasites in reptiles from Oman. GPS refers to the exact location where the animal was collected given in Table A.1 Numbers in bold indicate locations that were positive for Haemosporida.

Host species	*n*	Haemosporida		GPS
		Prevalence	Intensity %	
Assacus platyrhynchus	17			263,350
Bunopus spatalurus hajarensis	2			289
Calotes versicolor	1			274
Hemidactylus luqueorum	2	1 (50%)	0.18	**340**,350
Hemidactylus alkiyumii	1			277
Hemidactylus festivus	6			208,279
Hemidactylus hajarensis	3			289,319
Hemidactylus lemurinus	2			279
Messalina adramitana	2			284
Omanosaura jaykari	1			340
Pristurus carteri	11			205,268,278,284,286,287
Pristurus sp.1	2			278
Pseudotrapelus dhofarensis	1			205
Ptyodactylus hasselquistii	19	1 (5%)	0.34	208,263,289,292, **308**,326,339,340
Stenodactylus doriae	7			270,301
Stenodacylus leptocosymbotes	2			284
Trachylepis tessellata	1			274
	80	2 (2.5%)		

2.2. Molecular methods

DNA from the two samples that were identified as infected using microscopy was extracted from blood drops stored in Whatman filter paper stored at −20 °C using the Speedtools tissue DNA extraction kit (Biotools, Madrid), following manufacturer's instructions. PCR amplifications for a fragment of the mitochondrial cyt *b* gene were performed using the nested protocol with the outer primers HaemNFI [5′-CATA TAT-TAAGAGAAITATGGAG-3′] and HaemNR3 [5′ ATAGAAAGATAAGA AATACCATTC-3′] and the inner primers HAEMF (5′-ATGGTGCTTTCGAT ATATGCATG-3′) and HAEMR2 (5′-GCATTATCTGGATGTGATAATGGT-3′) [33]. The PCR reactions using these primers were run in 20 μl reaction mixture containing 1 U of GoTaq® DNA Polymerase (5 u/μl), 1.2 mM MgCl2 (25 mM), 0.125 mM of each nucleotide, 1× GoTaq® Flexi Buffer, 0.6 mM of each primer, and 2 μl of DNA. The reaction mix was heated to 94 °C for 3 min, and amplification was performed at 94 °C for 30 s, 50 °C and 54 °C, respectively, for 30 s, and 72 °C for 1 min, in 35 cycles, with a final 10 min extension at 72 °C. Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen Europe, Netherlands). The two obtained sequences were deposited in GenBank under accession numbers KT364883 (from *Hemidactylus luqueorum*) and KT364884 (from *Ptyodactylus hasselquistii*).

2.3. Phylogenetic analysis

Geneious v. R6.1.6 (Biomatters Ltd.) was used for assembling and editing the chromatographs. We performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) [39] to find the best match for the sequences against published sequences in GenBank. To produce an updated phylogeny of the cyt *b* gene of Haemosporida [25], we combined the information from published studies [10,28,35,40–43] (see Table A.2). Whole cyt *b* gene sequences were included when available, although the alignment consisted of different length sequences due to the discrepancies in sequencing between studies (total alignment length 1131 bp). A rooting approach that does not require an outgroup to be defined a priori was used, as this has been shown to be the most appropriate method to estimate the phylogenetic

Table 2
Microscopy measurements of Haemosporida intracellular parasites and infected host erythrocytes under 1000× magnification. *n* refers to the number of parasites or infected host cells measured per sample.

			Haemosporida — Mean ± Sd					Host cell — Mean ± Sd			
Host species	sex	code	*n*	Vertical	Horizontal	Area	Perimeter	*n*	Vertical	Horizontal	Area	Perimeter
Hemidactylus luqueorum	F	S7155	13	10.80 ± 0.87 (9.50–12.29)	4.33 ± 0.35 (3.85–5.05)	41.81 ± 3.54 (36.35–49.00)	29.69 ± 1.38 (27.78–32.00)	13	17.79 ± 1.23 (16.16–20.04)	10.75 ± 0.95 (8.76–11.99)	145.48 ± 14.63 (122.96–172.00)	49.24 ± 2.44 (44.69–53.00)
Ptyodactylus hasselquistii	M	S7668	12	11.61 ± 0.85 (9.85–12.84)	4.35 ± 0.53 (3.28–5.25)	41.65 ± 4.31 (35.12–52.18)	33.07 ± 1.85 (28.58–35.42)	12	19.06 ± 0.80 (17.79–20.55)	9.96 ± 0.69 (8.33–11.08)	154.86 ± 9.99 (141.65–170.89)	53.61 ± 2.08 (50.33–57.84)
			25	11.20 ± 0.86 (9.50–12.84)	4.34 ± 0.44 (3.28–5.25)	41.73 ± 3.92 (35.12–52.18)	31.38 ± 1.62 (27.78–35.42)	25	18.43 ± 1.02 (16.16–20.55)	10.02 ± 0.82 (8.33–11.99)	150.17 ± 12.31 (122.96–172.00)	51.43 ± 2.26 (44.69–57.84)

relationships between Haemosporida [25]. It has also been reported that the cyt *b* gene of these parasites often displays saturation in substitutions and biased frequencies at third-codon positions [44]. Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. We used PartitionFinder v1.1.0 [45] to infer the partition scheme and the model of sequence evolution for each partition with the following searching criteria: branchlengths = linked; models = raxml or models = mrbayes, depending on whether the output was used for ML or BI analyses; model_selection = BIC; three datablocks (one for each codon position of the cyt *b* gene); and search = all. The optimal gene partitioning scheme was all three codon positions together (single partition for the cyt *b* gene) and the selected model was the General Time Reversible, taking into account the shape of the gamma distribution and the number of invariant sites (GTR + G + I). Maximum Likelihood analysis was performed with RAxML v.7.0.3 [46], and reliability of the ML tree was assessed by bootstrap analysis [47] with 1000 replications. Bayesian Inference (BI) was performed using BEAST v1.7.5 [48]. Three independent runs of 5×10^7 generations were carried out, sampling at intervals of 10,000 generations, producing 5000 trees each. Models and prior specifications applied were as follows (otherwise by default): model of sequence evolution for the single cyt *b* partition GTR + I + G; Relaxed Uncorrelated Lognormal Clock; Yule birth death tree prior for the phylogenetic reconstruction; random starting tree; base substitution prior Uniform (0,100); and alpha prior Uniform (0,10). Posterior trace plots and effective sample sizes (ESS > 200) of the runs were monitored in Tracer v1.6 [49] to ensure convergence. The results of the individual runs were combined in LogCombiner discarding 10% of the samples and the consensus tree was produced with TreeAnnotator (both provided with the BEAST package). All trees were displayed in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The number of variable and parsimony-informative sites, and uncorrected *p*-distances were calculated in MEGA v6.06 [50].

3. Results and discussion

3.1. Phylogenetic relationships of Haemosporida based on the cyt b gene

The final cyt *b* dataset contained 309 sequences, composed of sequences of different lengths. The number of variable sites was 640 and of parsimony-informative sites 561. The BI and the ML approaches used in this study are similar regarding the topology and composition of most of the lineages and clades (Table A.1), although the ML analysis lacks support for most important nodes (Fig. 1). These poorly-supported nodes might be the result of using sequences of different lengths, which have been included because excluding missing data would require excluding many key taxa from the analyses. However, it has been shown that in general it is preferable to include some missing data rather than not including key taxa when estimating phylogenetic relationships [51]. Both estimates of phylogeny differ from the previous most complete analysis for cyt *b* gene [25] mainly in the position of the genera *Haemoproteus* (*Haemoproteus*), *Haemoproteus* (*Parahaemoproteus*) and *Leucocytozoon*. Based on our BI analysis, *Leucocytozoon* and *Haemoproteus* appear as sister taxa to avian and reptilian *Plasmodium*, in contrast with the aforementioned study in which these appear as derived lineages (Fig. 2). Our dataset contains sequences from genera that were underrepresented in that study, such as *Haemocystidium* and *Leucocytozoon* (*Akiba*), which may explain these differences in topology. These results corroborate the suggestion that *Leucocytozoon* is not appropriate for rooting the Haemosporida tree when using only the mitochondrial cyt *b* gene [10,25] because it branches within the Haemosporida group [24]. Therefore, depending on the phylogenetic analyses and taxon sampling used, these relationships are bound to change from study to study [9] and highlight that the current taxonomic status of Haemosporida may need revision. Our updated cyt *b* phylogeny corroborates the studies that show paraphyly of mammalian *Plasmodium* [10] (clades A–C) as a result of mammalian *Hepatocystis* (clade D) branching within it (Fig. 1), cryptic diversity within mammalian *Plasmodium* [52], polyphyly of bird *Plasmodium* [9,25,28], and the distinction between bird and reptilian *Haemoproteus*, with monophyly of reptilian *Haemocystidium* [35]. Therefore, based on the cyt *b* gene both avian and reptilian *Plasmodium* are polyphyletic as lineages occur in different parts of the tree with unresolved relationships with other genera, such as *Polychromophilus* from chiropteran hosts (clade O). Interestingly, sequence EF179355 identified as an unknown Haemosporida and obtained from the lesser false vampire bat (*Megaderma spasma*) from Cambodia (lineage M) [40] is placed inside reptilian *Plasmodium* clades (clades L and N, Fig. 1; see supplementary Figs. A.1 and A.2). This lineage poses important questions for transmission dynamics of these parasites, such as: is this a new and underrepresented clade of haemosporidians of bats? Is it derived from *Polychromophilus* or has it arisen from horizontal gene transfer from an avian and/or reptilian host?

Regarding the reconstruction of the evolutionary history of Haemosporida based on the cyt *b* gene, our results corroborate the fact that life-history traits do not seem to have played a major role in major cladogenetic events within this order [10]. In our phylogeny, there has been a loss of merogony in the ancestral form that gave rise to avian *Leucocytozoon* and *Haemoproteus* and the ancestral form of avian and reptilian *Plasmodium* regained this trait. In contrast, the major cladogenetic events seem to be associated with vector host shifts as previously proposed [10], with vector host families associated with the main clades (Figs. 1 and 2). Moreover, some studies show that host identity may be an invalid taxonomic character in avian hosts due to frequent host-shifts and host-sharing of Haemosporida (e.g. *Haemoproteus* spp. [53–55]). Our results show that this might be particularly frequent for avian and reptilian *Plasmodium* (Fig. 2). Therefore, when the vector host diversity and specify are better understood, an alternative to the current parasite naming system of haemosporidians would be to take into account the vector host. Regarding reptilian *Haemocystidium*, the chelonian haemosporidians *Haemocystidium* (*Simondia*) may be transmitted by tabanid flies (Tabanidae), however the vectors of *Haemocystidium* (*Haemocystidium*) that infect squamate hosts are still unknown [35]. Our estimate of phylogeny is solely based on a single mitochondrial gene. A recent study that included several nuclear genes found alternative relationships, such as *Leucocytozoon* was basal to *Haemoproteus* and *Plasmodium* or that mammalian *Plasmodium* was sister taxa to bird and reptile *Plasmodium* [59].

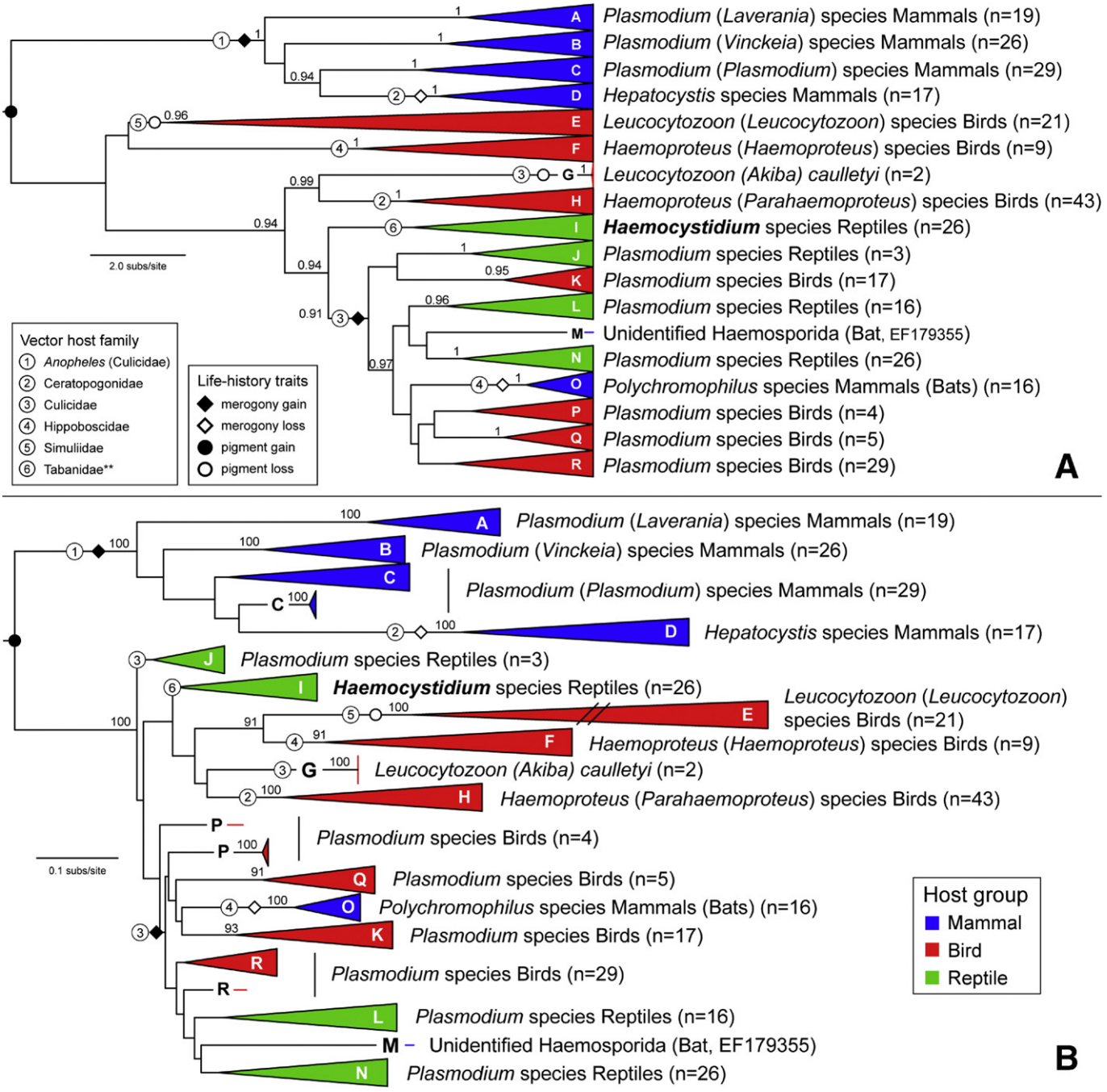

Fig. 1. Trees derived from Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of the cyt *b* gene of Haemosporida. A) BI using a Relaxed Uncorrelated Lognormal Clock prior that places the root between mammalian *Plasmodium*/*Hepatocystis* and all other parasite lineages. Bayesian Posterior Probability values above 0.90 are shown by the nodes. B) ML rooted between mammalian *Plasmodium*/*Hepatocystis* and all other parasite lineages (following the results of the BI analysis). Maximum Likelihood bootstrap values above 70 are given above relevant nodes. In the ML analysis (B) the *Leucocytozoon* clade was shortened by 50%. Diamonds indicate merogony gain or loss and circles indicate hemozoin pigment gain or loss [25]. Numbers inside circles indicate the host vector family [9,10]. **Tabanidae are known vectors of *Haemocystidium* (*Simondia*) that infect North American chelonians, but the vectors of squamate *Haemocystidium* (*Haemocystidium*) are still unknown [9]. Colors indicate the vertebrate host. The new sequences from this study are placed in the highlighted *Haemocystidium* clade (see Table A.2, Figs. A.1 and A.2 for more details).

Finally, we show some potential misidentifications of sequences deposited in GenBank. This growing problem was already known (e.g. AF069613 that was originally identified as *Haemoproteus columbae*, appears to be a *Plasmodium* species [56] and was corroborated in our study (clade Q, Figs. A.1 and A.2)). For instance, a sequence previously identified as *Plasmodium* sp. from the lizard *Egernia stokesii* (EU254531) is placed in the *Haemocystidium* clade (see Supplementary Figs. A.1 and A.2). This misidentification may have been due to a lack of taxon sampling of reptile *Haemocystidium* at the time of the study [10,44]. Moreover, sequences identified as *Plasmodium relictum*, the most common *Plasmodium* species in birds, appear in different lineages within clade R of bird malaria (e.g. EU254538 and AY733090). To reduce this kind of misidentifications, the database MalAvi [57] was created in order to better characterize the diversity of avian Haemosporida. Although reptilian Haemosporida are still relatively understudied in comparison to bird Haemosporida, it would be important to create a similar platform for reptilian parasites.

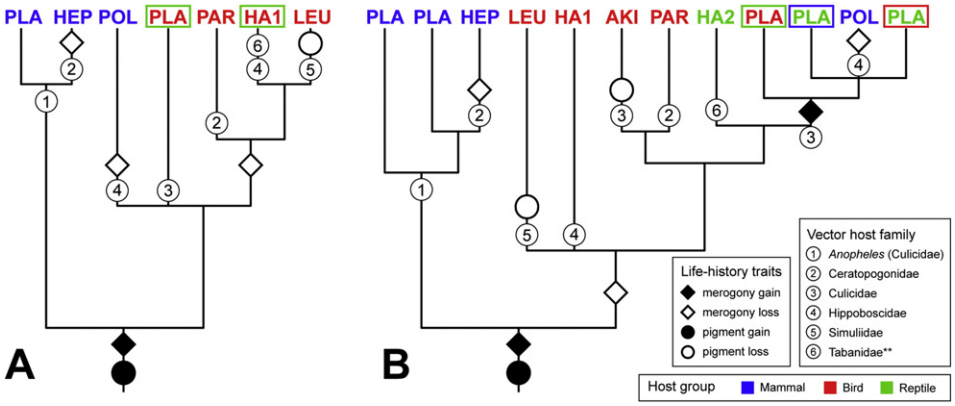

Fig. 2. Topologies for the evolution of Haemosporida based on cyt *b* under a relaxed molecular clock. A) Adapted from Outlaw and Ricklefs (2011) topology. B) Most updated enlarged cyt *b* gene dataset. PLA, *Plasmodium*; HEP, *Hepatocystis*; POL, *Polychromophilus*; PAR, *Parahaemoproteus*; HA1, *Haemoproteus*; HA2, *Haemocystidium*; LEU, *Leucocytozoon*; AKI, *Akiba*. Colored rectangles indicate a smaller fraction of the corresponding host group that is also found in this clade (mainly composed of the host colors in the letters). Diamonds indicate merogony gain or loss and circles indicate hemozoin pigment gain or loss [25] and numbers inside circles indicate the host vector family [9,10] (see Fig. 1).

3.2. Characterization of *Haemocystidium* in geckos from Oman

Of the 80 samples screened with microscopy, only 2 (3%) were infected with Haemosporida. Infected individuals were from northern populations (*P. hasselquistii* from 22.7914 N, 59.22873 E and *H. luqueorum* from 23.18292 N, 57.41627 E, see Tables 1 and A.1 for more information). Intensity of Haemosporida was low, with less than 14 gametocytes per 4000 erythrocytes (Table 1). Microscopic examination of Haemosporida stages in the blood of the two gecko hosts showed ovoid young gametocytes inside erythrocytes (Fig. 3) [17]. Gametocytes were small, occupying about a quarter of the erythrocyte area (Table 2), without considerably changing the cell morphology (Fig. 3). Most gametocytes stained light pink, with some displaying darker pigments (Fig. 3). These characteristics resemble those of young *Haemocystidium* stages in chelonians [35, 43] and lizards [58].

The cyt *b* sequences obtained for these two samples were placed in a clade that is exclusively composed of haemosporidians of reptiles (clade I, Fig. 1, Supplementary Figs. A.1 and A.2) and that was recently reclassified as *Haemocystidium* based on morphological and genetic data in comparison to avian *Haemoproteus* (clades F and H, Fig. 1, Supplementary Fig. A.1) [35]. The aforementioned study was the first to assess the molecular diversity of reptilian *Haemocystidium* and showed the uncertainty of the classification of these parasites. In our BI analysis of the cyt *b* gene, the *Haemocystidium* clade is sister taxa to reptile and bird *Plasmodium* and mammalian *Polychromophilus* (clades J–R, Fig. 1A) and all these are sister taxa to bird *Haemoproteus* (*Parahaemoproteus*) (clade H, Fig. 1A) and *Leucocytozoon* (*Akiba*) *caulletyi* (clade G, Fig. 1A).

The two new sequences from Oman were genetically identical. The closest match on GenBank to our two new sequences was from *Haemocystidium* sp. from the collared iguana *Oplurus cuvieri* (DQ212191) from Madagascar, with an uncorrected *p*-distance of 1.9% (Table 3). The other estimates of sequence divergence for the cyt *b* gene show that our sequences diverge by 6–7% from *Haemocystidium* species from other reptiles (Table 3). The cyt *b* gene has been the most widely used gene for identifying new species, for which the generally accepted cut-off has been above 5% divergence [31]. Finding similar Haemosporida in two gecko species belonging to two different families, *H. luqueorum* (Gekkonidae) and *P. hasselquistii* (Phyllodactylidae) is further evidence that describing new species based on identification in new host species without further information is not a good practice [18]. Indeed, *P. hasselquistii* may have at least two different *Haemoproteus* species, that is, *Haemoproteus ptyodactylii* and that reported in this study. Based on the fact that the new haemosporidian sequences differ from named *Haemoproteus* species by more than 5% in cyt *b*, it is reasonable to assume that these may represent a new species of *Haemoproteus* that is currently found in geckos from Oman and possibly also in an iguanid lizard from Madagascar. However, the determination of the genetic diversity within this putative new species is needed, as well as the identification of the vectors.

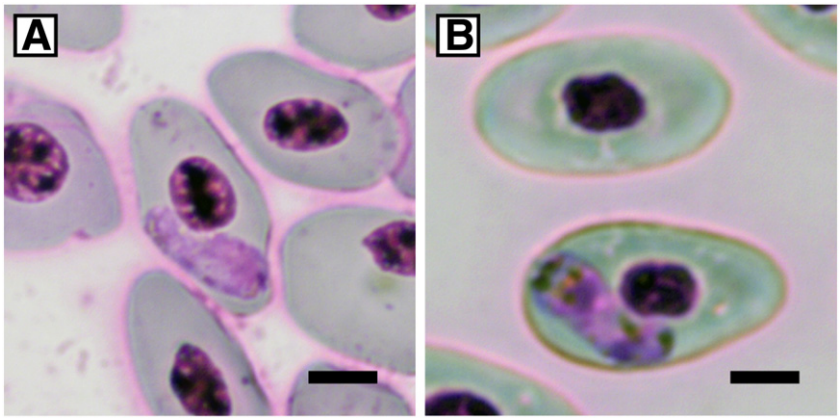

Fig. 3. Haemosporida parasites found in reptile hosts from Oman. *Haemocystidium* sp. from *Hemidactylus luqueorum* (sample S7155, Fig. 3); and from *Ptyodactylus hasselquistii* (sample S7688, Fig. 3). Scale bar is 5 μm.

Table 3
Estimates of evolutionary divergence between selected species of Haemosporida and the haplotype retrieved in this study. The number of base differences per site from between sequences are shown in ascending order for the cyt *b* gene. Refer to Table A.3 for references.

Parasite species	Code	*p*-Distance	Host species	Geographic locality	Ref
Haemocystidium sp.	S7155	–	*Hemidactylus luqueorum*	Oman	Here
Haemocystidium sp.	S7668	–	*Ptyodactylus hasselquistii*	Oman	Here
Haemocystidium sp.	DQ212191	0.019	*Oplurus cuvieri*	–	Unpub
Haemocystidium pacayae	KF049495	0.059	*Podocnemis expansa*	Peru	[33]
Haemocystidium ptyodactylii	AY099057	0.069	*Ptyodactylus hasselquistii*	Israel	[28]
Haemocystidium kopki	AY099062	0.073	*Teratoscincus scincus*	Pakistan	[28]
Haemocystidium mesnili	KF049514	0.073	*Naja annulifera*	South Africa	[33]
Haemocystidium sp.	EU254531	0.074	*Egernia stokesii*	Australia	[10]
Haemocystidium anatolicum	JQ039742	0.077	*Testudo graeca*	Turkey	[43]
Haemocystidium peltocephali	KF049491	0.085	*Podocnemis expansa*	Peru	[33]

4. Conclusion

Our study presents a reconstruction of the evolutionary history of Haemosporida for the cyt *b* gene and corroborates the need for a taxonomic revision of this order, as well as the need for more taxon sampling, especially regarding reptilian haemosporidians. Phylogenetic relationships may change based on the gene(s) used, therefore future studies should include both mitochondrial and nuclear genes of all genera within Haemosporida. The newly detected *Haemocystidium* sp. in geckos from Oman may represent a different strain or a possible new species that needs to be further sampled and the vectors identified. Our results also exemplify the impact of taxon sampling biases on the phylogenetic relationships of the group based on the cyt *b* gene that are important for understanding their evolutionary history. The observation that major cladogenic events seem to be associated with shifts between vector host families highlights the need for identification of the vectors and the study of the vector stages in underrepresented taxa, such as *Haemocystidium*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2015.09.003.

Acknowledgements

We thank Elena Gómez-Díaz for participating in the 2011 fieldtrip to Oman and for her help revising an early version of the manuscript. We are indebted to Ali Alkiyumii and the other members of the Ministry of Environment and Climate Affairs of the Sultanate of Oman for their help and support and for issuing all the necessary collecting and exporting permits (Refs: 12/2011), to Felix Amat for participating in the 2011 fieldtrip to Oman, and to Michael Robinson for logistic support in Oman. JPM was funded through a PhD grant (SFRH/BD/74305/2010) supported by a Fundação para a Ciência e a Tecnologia (FCT) doctoral fellowship under the Programa Operacional Potencial Humano — Quadro de Referência Estratégico Nacional funds (POPH-QREN) from the European Social Fund (ESF) and Portuguese Ministério da Educação e Ciência. DJH is supported by FEDER through the compete program, the project "Genomics and Evolutionary Biology" cofinanced by North Portugal Regional Operational Program (ON.2) under NSRF through the European Regional Development Fund. SC is supported by grant CGL2012-36970 from the Ministerio de Economía y Competitividad, Spain (co-funded by FEDER). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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