Haemogregarines from western Palaearctic freshwater turtles (genera *Emys, Mauremys*) are conspecific with *Haemogregarina stepanowi* Danilewsky, 1885

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SUMMARY

The majority of *Haemogregarina* species have been based on the morphology of their erythrocytic stages and supposed strict host specificity. The quantity of species with a limited number of overlapping diagnostic traits has led to a considerable mess in haemogregarine taxonomy and significant synonymy. We analysed host specificity, intra- and interspecific variability, evolutionary relationships, and the distribution of the type species of the genus *Haemogregarina* – *H. stepanowi*. The morphology of blood stages and 18S rDNA sequences of this haemogregarine from four western Palaearctic hard-shelled freshwater turtles (*Emys orbicularis, Mauremys caspica, Mauremys leprosa* and *Mauremys rivulata*) were compared with *Haemogregarina balli*. Additional sequences of 18S rDNA of *Haemogregarina*-like isolates collected from three species of African hinged terrapins (genus *Pelusios*) were used to enlarge the dataset for phylogenetic analyses. Thirteen sequences (1085 bp) of *Haemogregarina* representing all four western Palaearctic turtle species were identical, corresponding to *H. stepanowi*, which is closely related to the Nearctic species *H. balli*. In our analyses, *Haemogregarina* spp. constituted a monophyletic clade sister to the genus *Hepatozoon*. *Haemogregarina stepanowi* possesses a wide distribution range from the Maghreb, through Europe, Turkey and the Middle East to Iran. We consider that the genus *Haemogregarina* has a low host specificity crossing the family level of its vertebrate hosts and that its distribution is likely to be linked to the vector and definitive host – the leech.

Key words: Apicomplexa, *Haemogregarina*, *Emys*, *Mauremys*, *Pelusios*, 18S rDNA, phylogenetic analysis, host specificity.

INTRODUCTION

Haemogregarines form a group of about 400 species of adeleid blood parasites with a suspected heteroxenous life cycle, species of the genus *Haemogregarina* being primarily parasites of reptiles and fish (Desser, 1993). In the past, the majority of new *Haemogregarina* species were described based on the morphology of their erythrocytic stages – e.g. gamonts and meronts. Moreover, strict host specificity was supposed, so each new studied host species led to a description of a new parasite. Such a practice led to considerable synonymy. In his review, Levine (1988) placed 300 species into the genus *Haemogregarina*, whereas Siddall (1995) designated only 19 to represent *Haemogregarina* sensu stricto. The biology, vectors and transmission routes of haemogregarines are mostly unknown (Desser, 1993). Complete life cycles, allowing for taxonomic conclusions, have been described for only two *Haemogregarina* species of turtles: the type species of the genus, the parasite of the European pond turtle (*Emys orbicularis*) – *Haemogregarina stepanowi* (see Danilewsky, 1885; Reichenow, 1910); and for the parasite of Nearctic snapping turtles (*Chelydra serpentina*) – *Haemogregarina balli* (see Paterson and Desser, 1976; Siddall and Desser, 1990, 1992). In both haemogregarine species, sexual development occurs in the leech hosts, where gametogenesis, the formation of the zygote and the monosporoblastic oocyst occur within intestinal epithelial cells. Subsequent merogony in

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endothelial cells produces hundreds of merozoites, invading the proboscis of the leech and inoculating the turtle during feeding. Pre-erythrocytic merogony takes place in the lungs, liver and spleen of the turtle. The last part of the life cycle comprising secondary merogony and the formation of gamonts occurs in the erythrocytes of the turtle, allowing for relatively easy detection (Reichenow, 1910; Paterson and Desser, 1976; Siddall and Desser, 1990, 1992).

The Western Palaearctic is inhabited by four species of hard-shelled freshwater turtles belonging to two families – *E. orbicularis* (Emydidae), *Mauremys caspica, Mauremys rivulata* and *Mauremys leprosa* (Geoemydidae). As well as *H. stepanowi* from *E. orbicularis* (see Mihalca *et al*. 2008), the occurrence of haemogregarines has been reported in the blood of *M. rivulata* (see Desser and Yekutiel, 1987; Paperna, 1989), albeit without taxonomic considerations. Additionally, *Haemogregarina bagensis* (Ducloux, 1904) was described from *M. leprosa* from north-western Tunisia. Subsequently, Billet (1904) described two forms of this parasite in *M. leprosa* from Algeria. The description of this haemogregarine from this same host was completed by Laveran and Pettit (1909). Surprisingly, more than a hundred years after their description, very little is still known about the biodiversity, taxonomy, evolutionary relationships and biogeography of these turtle haemogregarines.

The development of modern molecular-genetic methods has provided new, powerful tools to enable the acceptance or refutation of the validity of numerous previously described species and to study their genealogy and evolution. Surprisingly, these simple methods have still not been widely applied in the taxonomy of haemogregarines (see e.g. Wozniak *et al*. 1994), which still awaits revision. Even recent descriptions of new species frequently lack molecular-genetic specification and genealogical placement within the evolutionary tree of adeleids. Among *Haemogregarina* species, only *H. balli* phylogeny has been studied using molecular-genetic tools (Barta *et al*. 2012).

By sampling all western Palaearctic hard-shelled freshwater turtle species from a wide geographical range, we set the following objectives for this study: (1) to test the relationships between *H. balli* and *H. stepanowi* by comparing their 18S rDNA sequences and to confirm their congeneric status within the genus *Haemogregarina*; (2) to evaluate the conspecificity of *Haemogregarina* isolates from all four western Palaearctic turtle species (genera *Emys* and *Mauremys*) by comparing the morphology of their blood-stages and by phylogenetic analyses of 18S rDNA sequences; (3) to consider the host specificity of *Haemogregarina* at the level of their vertebrate intermediate host.

MATERIALS AND METHODS

Sampling

Eighty-one turtles were collected by hand during 2005–2013 in Morocco, north-eastern Algeria, southern Bulgaria, Turkey, western Syria and Iran (Table 1). With a few exceptions, the animals were classified as male, female and juvenile. All turtles were released immediately afterwards at the location of capture. Blood samples were taken by puncture of the dorsal coccygeal vein and fixed in 96% pure ethanol. Thin blood smears were air dried, fixed in absolute methanol for 5 min and stained with Giemsa diluted 1 : 10 in distilled water (pH 7) for 20 min. The smears were examined by light microscopy using a 100× magnification objective lens equipped with immersion oil. Images were captured by Olympus DP 73 digital camera. For each infected turtle, the intensity of parasitaemia was estimated as the percentage of infected erythrocytes present in approximately 10^4 cells (e.g. 100 fields each with estimated 100 erythrocytes per field).

DNA extraction, PCR, sequencing

DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's protocol and eluted in 100 μL of PCR water. Extracted DNA was quantified spectrophotometrically using a Nanodrop ASP-3700 (ACTGene, USA) and then stored at −20 °C. A pair of specific primers (EF: 5′-GAAACTGCGAATGGCTCATT-3′ and ER: 5′-CTTGCGCCTACTAGGCATTC-3′) designed originally for *Eimeria* by Kvičerová *et al*. (2008) was used to confirm the presence of DNA of apicomplexan parasites, amplifying up to 1500 bp long fragments of nuclear 18S rDNA of *Haemogregarina*. This gene proved to be sufficiently variable and informative both on the generic and specific levels within Apicomplexa (Barta *et al*. 2012). PCR reactions were carried out in a 25 μL volume, including 1 μL each of 10 μM PCR primer, 12·5 μL of Combi PPP Master Mix (Top-Bio, Czech Republic), 8·5 μL of PCR water and 2 μL of extracted DNA.

PCR conditions were as follows: initial denaturation at 95 °C for 4 min, followed by 30 cycles consisting of denaturation at 92 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The results of amplification were visualized on 1·2% agarose gel using ethidium bromide under UV light. PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan). The concentration of DNA was then measured. DNA sequencing was provided by the service laboratory (Macrogen Inc., the Netherlands) on an automatic 3730XL DNA analyser.

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Table 1. Studied material; m – males, f – females, juv – juveniles, nd – not detected

Host	Collection site	Number m/f/juv/nd
Emys orbicularis (Linnaeus, 1758)	Bulgaria: Zhelezino	4/3/1/0
E. orbicularis	Iran: Mazandaran province	2/0/0/0
E. orbicularis	Iran: Golestan p.: Ziarat village	0/1/0/0
E. orbicularis	Turkey: Eastern Anatolia region: Diyarbakir	0/0/0/4
Mauremys caspica (Gmelin, 1774)	Iran: Golestan p.: 30 km E of Gorgan: Taghi Abad village	1/0/0/0
M. caspica	Iran: Golestan p.: E of Agh Ghala – sturgeon fish culture centre	0/1/0/0
M. caspica	Iran: Mazandaran p.: Sari-Tajeddin: Mahalleh village	1/0/0/0
M. caspica	Iran: Khuzestan p.: Shadegan city	0/0/2/0
M. caspica	Iran: Kermanshah-Gamasiab river	1/2/0/0
M. caspica	Turkey: Eastern Anatolia region: Diyarbakir	0/0/0/9
Mauremys leprosa (Schweigger, 1812)	Algeria: 10 km to west from Annaba	2/4/0/0
M. leprosa	Morocco: Oulmes	0/0/1/0
M. leprosa	Morocco: Tnine de l'Ourika	1/0/0/0
Mauremys rivulata (Valenciennes, 1833)	Bulgaria: Zhelezino	3/0/0/0
M. rivulata	Turkey: Marmara region: Balikesir	1/0/1/0
M. rivulata	Turkey: Aegean region: Selçuk	2/1/1/0
M. rivulata	Turkey: Aegean region: Kemer (Fethiye)	1/3/1/0
M. rivulata	Syria: Jourine	1/1/0/0
M. rivulata	Syria: channel between Jourine and Orontes	1/0/0/0
M. rivulata	Syria: Orontes	1/1/4/0
M. rivulata	Syria: Masyaf	1/1/2/0
M. rivulata	Syria: Einl Taqa	3/7/1/0
M. rivulata	Syria: Mazraet Mardido: Al Qash river	0/1/2/0

Phylogenetic analyses

Obtained sequences were identified by BLAST analysis, edited using the DNASTAR program package (DNASTAR Inc.) and deposited to the NCBI GenBank database under the accession numbers KF257926, KF257927, KF257928 and KF257929. Additionally, selected sequences from members of genera *Adelina*, *Babesiosoma*, *Cryptosporidium*, *Dactylosoma*, *Haemogregarina* and *Hepatozoon* were obtained from the GenBank (NCBI) (Table 2). Since only a single sequence of the genus *Haemogregarina*, namely *H. balli* (Barta *et al*. 2012), was available in the GenBank, we enlarged the dataset by adding sequences of three *Haemogregarina*-like parasites collected from three African terrapins of the genus *Pelusios* available in our lab – *P. marani* from Gabon (KF257924), *P. williamsi* from Kenya (KF257923) and *P. subniger* from Mozambique (KF257925) (Table 2). The sequences were aligned in BioEdit (Hall, 1999) using the ClustalW algorithm (Thompson *et al*. 1994). MEGA 5.0 (Tamura *et al*. 2011) was used to calculate genetic distances based on 18S rDNA sequences of *Haemogregarina* included in the analyses. Bayesian inference analysis (BI) was carried out with Mr Bayes 3.1.2. using a GTR + Γ + I model for 10 million generations (Ronquist and Huelsenbeck, 2003). Chain convergence and burn-in were estimated according to the indices implemented in the MrBayes program (deviation of split frequencies, potential scale reduction factor – PSRF) and using a Tracer program (Rambaut and Drummond, 2007). The trees were summarized after removing burn-in (600 trees). Maximum likelihood analysis (ML) was performed in PHYML 2.4.4. (Guindon and Gascuel, 2003), with the GTR + Γ + I model and parameters estimated from the data; bootstrap values were calculated for 1000 replicates. Resulting trees, including *Cryptosporidium serpentis* as an outgroup [AF093499], were visualized using TreeView 1.6.6. (Page, 1996).

RESULTS

Microscopy

Altogether, 47/81 (58·0%) of turtles were infected with *Haemogregarina* sp. (Table 3). The highest prevalence occurred in *E. orbicularis*, where 13/15 turtles (86·7%) were parasitized. Among turtles of the genus *Mauremys*, 8/17 *M. caspica* (47·1%), 2/8 *M. leprosa* (25·0%), and 24/41 *M. rivulata* (58·5%) were infected with *Haemogregarina* sp. Parasitaemia in infected individuals reached 0·08–1·36%. We observed four main developmental stages as reported for *Haemogregarina* in turtle hosts by Telford (2009), namely intraerythrocytic trophozoites, premeronts, meronts and gamonts (see Fig. 1), including their intermediate forms. Gamonts were the most frequently observed, followed by premeronts and trophozoites. Meronts were the rarest (Table 4).

Trophozoites were the smallest life-stages. They were slightly curved in shape with a nucleus located

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Table 2. The GenBank accession numbers of the sequences included in the phylogenetic analyses

Organism	Acc. number	References
Adelina dimidiata	DQ096835	Kopečná *et al*. (2006)
Cryptosporidium serpentis	AF093499	Xiao *et al*. (1999)
Dactylosoma ranarum	HQ224958	Barta *et al*. (2012)
Haemogregarina balli	HQ224959	Barta *et al*. (2012)
Haemogregarina sp. (*P. marani*, Gabon)	KF257924	This study
Haemogregarina sp. (*P. subniger*, Mozambique)	KF257925	This study
Haemogregarina sp. (*P. williamsi*, Kenya)	KF257923	This study
Haemogregarina stepanowi (*E. orbicularis*, Bulgaria)	KF257928	This study
Haemogregarina stepanowi (*M. caspica*, Iran)	KF257926	This study
Haemogregarina stepanowi (*M. leprosa*, Algeria)	KF257929	This study
Haemogregarina stepanowi (*M. rivulata*, Syria)	KF257927	This study
Babesiosoma stableri	HQ224961	Barta *et al*. (2012)
Hepatozoon sp. *Boiga*	AF297085	Jakes *et al*. unpublished
Hepatozoon americanum	AF176836	Mathew *et al*. (2000)
Hepatozoon ayorgbor	EF157822	Sloboda *et al*. (2007)
Hepatozoon canis	AY461378	Criado-Fornelio *et al*. unpublished
Hepatozoon catesbianae	AF130961	Carreno *et al*. (1999)
Hepatozoon clamatae	HQ224962	Barta *et al*. (2012)
Hepatozoon felis	AY620232	Criado-Fornelio *et al*. (2006)
Hepatozoon sipedon	JN181157	Barta *et al*. (2012)
Hepatozoon ursi	EU041717	Kubo *et al*. (2008)

close to one end, and their cytoplasm contained a large number of vacuoles (Fig. 1a, e, i and m). Encapsulated premerонts were more elongated, slightly curved, and, compared with trophozoites, they lacked vacuoles. Their nuclei were usually located in the central position (Fig. 1b, f, j and n). Erythrocytic meronts containing multiple nuclei were rarely found (Fig. 1c, g and k). The prevailing gamonts laid in erythrocytes in a bean-shaped capsule. They were recurved, and with the majority showing a central brightening (Fig. 1d, h, l and o). The stained deep purple nucleus was located near or directly at the bend of the parasite. Young forms of gamonts had nuclei with stranded chromatin, but in mature forms the nucleus shape was dependent on its position. Infected erythrocytes were wider and their nucleus was displaced to the edge of the cell; a few erythrocytes contained two gamonts, two premerонts (Fig. 1p), or a gamont together with a premeront. For each host species, the measurements of all developmental stages are reported in detail in Table 4. The observed morphology of all forms fitted well the morphology of *H. stepanowi* as described by Danilewsky (1885) and Reichenow (1910), thus our isolates were considered to represent this species. Additionally, a considerable overlap within morphological traits further suggested the conspecificity of isolates from all four western Palaearctic hard-shelled freshwater turtle species.

Phylogenetic analyses

PCR analysis offered the same sensitivity as microscopy; all microscopically *Haemogregarina*-positive samples yielded corresponding PCR products. 18S rDNA sequences were obtained from 19/47 *Haemogregarina*-positive samples, the lengths of which ranged from 737 and 1421 bp. We used the 13 longest sequences for subsequent analyses. Alignment along the 1085 bp overlapping region was identical for all samples (for *p* distances between studied taxa, see Table 5). Four sequences of *H. stepanowi* (one for each host species) were included in the phylogenetic analyses. Resulting phylogenetic trees provided identical topology for both BI and ML analyses (Fig. 2). Analysed haemogregarines formed a monophyletic cluster with three main branches: (i) a clade of sequences of *Dactylosoma ranarum* as a sister taxon of *Babesiosoma stableri*; (ii) a large clade of *Hepatozoon* sp. sequences, divided into two well-separated groups, one being composed of mammalian species of *Hepatozoon*, and a second formed by *Hepatozoon* species from reptiles and amphibians; and (iii) a clade consisting of sequences of *Haemogregarina*. The Nearctic species, *H. balli*, represented a sister taxon to our four isolates of *H. stepanowi*, while isolates of *Haemogregarina*-like parasites from African hinged terrapins formed a sister clade to *H. balli* and *H. stepanowi*. All nodes were well-resolved and highly supported. Our analyses revealed the evident conspecificity of all our isolates from *E. orbicularis* and *Mauremys* spp. and their affiliation to the genus *Haemogregarina* together with *H. balli* and with haemogregarines from African hinged terrapins.

DISCUSSION

Apicomplexans are among the most tangled organisms in respect to their phylogenetic reconstructions.

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Table 3. Numbers of collected turtles and prevalence of infection; m – males, f – females, juv – juveniles, nd – not detected

Host tortoises	m/f/juv/nd	Prevalence by hosts and countries: examined/infected (%)						Total
		Bulgaria	Syria	Iran	Turkey	Morocco	Algeria	
E. orbicularis	6/4/1/4	8/8 (100)	–	3/1 (33·3)	4/4 (100)	–	–	15/13 (86·7)
M. caspica	3/3/2/9	–	–	8/5 (62·5)	9/3 (33·3)	–	–	17/8 (47·1)
M. leprosa	3/4/1/0	–	–	–	–	2/0 (0)	6/2 (33·3)	8/2 (25·0)
M. rivulata	14/15/12/0	3/3 (100)	27/17 (63·0)	–	11/4 (36·4)	–	–	41/24 (58·5)
Total	26/26/16/13	11/11 (100)	27/17 (63·0)	11/6 (54·5)	24/11 (45·8)	2/0 (0)	6/2 (33·3)	81/47 (58·0)

Their perceived evolutionary relationships can be distorted in some instances by the lack of distinct morphological features at the light microscopy level. Our knowledge of the phylogeny of Apicomplexa has considerably increased with the expansion of molecular methods based on a variety of markers. However, though medically and veterinary important groups of Apicomplexa have become frequently studied via these methods, others have been largely overlooked. With the exception of the genus *Hepatozoon*, such neglect applies to haemogregarines of the suborder Adeleorina.

The type species of the genus *Haemogregarina*, *H. stepanowi*, has been frequently studied, however, without emphasis on its proper generic allocation, genealogy, and phylogeny with respect to other haemogregarines. Moreover, it has also been formerly reported from various Nearctic turtles, such as *Emydoidea blandingii*, *Chrysemys picta*, and *C. serpentina* (Hahn, 1909; Roudabush and Coatney, 1937). The latter is the host of *H. balli* – the best studied of the *Haemogregarina*. Our phylogenetic analyses based on partial 18S rDNA sequences confirmed the close relationships between *H. stepanowi* and *H. balli*, however, always as two individual species. Both applied methods – Bayesian inference and maximum likelihood – confirmed their affiliation to the genus *Haemogregarina* together with three studied isolates of haemogregarines from African hinged terrapins.

The size and other morphological traits of developmental stages of *Haemogregarina* available in the peripheral blood of all four studied Palaearctic turtles considerably overlap, possessing low variability. They correspond well with the morphology of the appropriate stages of *H. stepanowi* as described by previous authors (Danilewsky, 1885; Reichenow, 1910; Telford, 2009). However, slight morphological differences are present. In particular, haemogregarines found in *M. leprosa* resembled small 'endoglobular' (cylindrical with round edges, possessing a nucleus composed of a large amount of chromatin) and 'vermicular' (recurved with a nucleus located at the bend) forms of *H. bagensis* as reported by Billet (1904), Ducloux (1904) and Laveran and Pettit (1909). This necessitates comparison of *H. stepanowi* with *H. bagensis*, since the latter was considered by Siddall (1995) as a valid species. The morphology of the individual stages is very similar and their mutual comparison is also made difficult by the uneven quality of old descriptions. Only Laveran and Pettit (1909) provided dimensions of premeronts, gamonts and erythrocytic meronts of *H. bagensis* comparable to those of *H. stepanowi* (Table 4). The size of premeronts of the two haemogregarines is slightly different, *H. bagensis* being more slender. A complication arises in gamonts, because Laveran and Pettit (1909) did not consider the recurving of the parasite within the erythrocyte. Nevertheless, taking this into account, their size is similar. The dimensions

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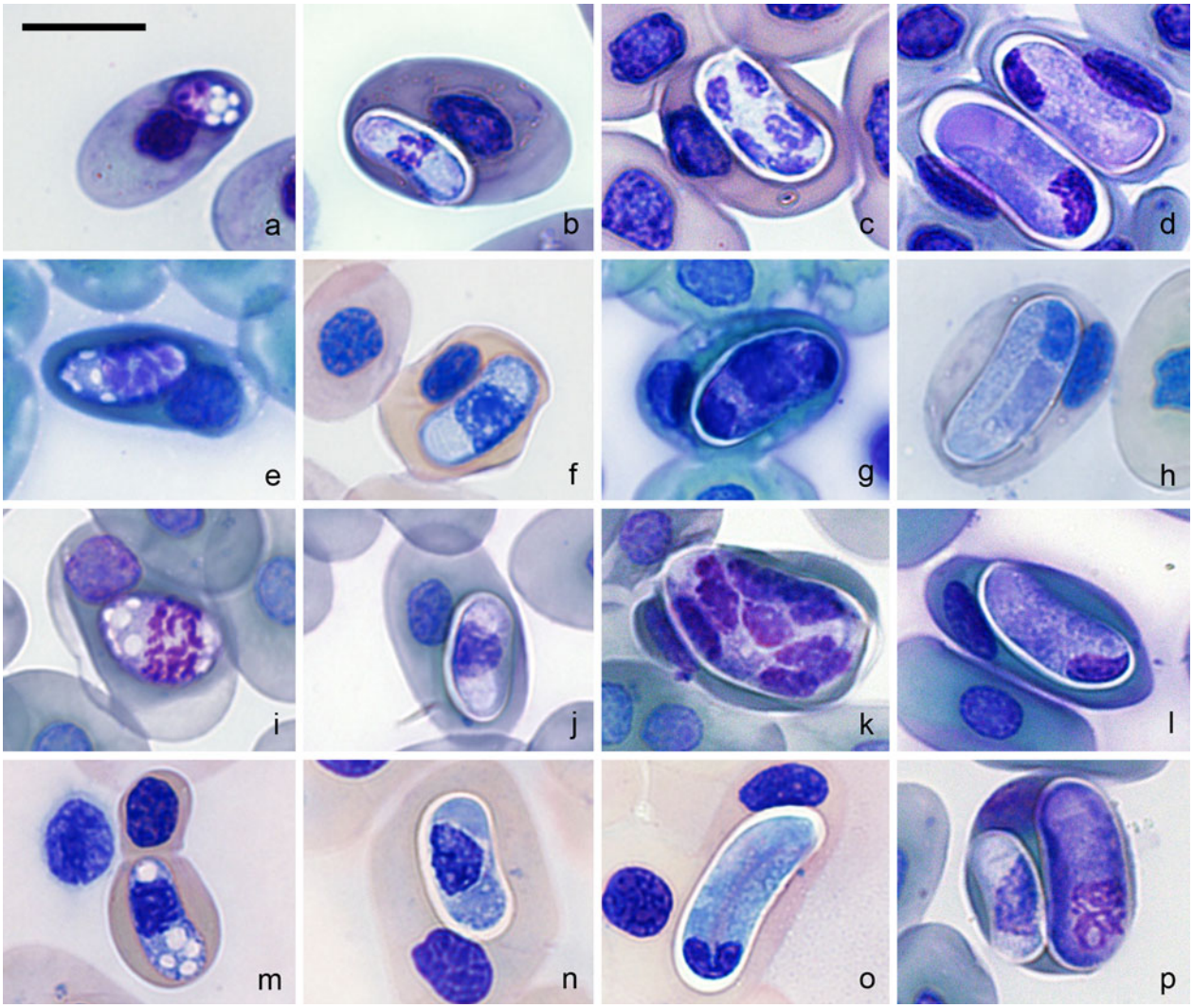

Fig. 1. Endogenous life stages of *Haemogregarina stepanowi* from *Emys orbicularis* (a–d), *Mauremys caspica* (e–h, p), *M. rivulata* (i–l), and *M. leprosa* (m–o), all in the same scale. Trophozoites contain numerous vacuoles (a, e, i, m), premerонts possess the nucleus in a central position and lack the vacuoles (b, f, j, n), meronts of variable sizes possess various number of nuclei (c, g, k), gamonts are curved in a capsula, with nucleus located at the bend (d, h, l, o), premerонts of different developmental stages in a single erythrocyte of *M. caspica* (p). Scale bar 10 μm.

of erythrocytic meronts vary depending on the number of nuclei; 4–16 nuclei being reported in *H. bagensis* and 2–24 nuclei being reported in *H. stepanowi*. Therefore, the size of meronts is not a reliable tool for comparison among the species of haemogregarines. We suppose that slight differences in morphology of blood stages (Table 4) might be attributed to their intraspecific variability, to the various phases of infection, to the influence of different host species, and to the different measuring methods used by former authors.

The prevalence of individual stages in the blood-stream depends on the duration of infection, when long-term infection can be characterized by a higher number of gametogonic and merogonic forms, and by fewer trophozoites (Mihalca *et al*. 2002). The old infections are characterized by prevailing gamonts in the peripheral blood. This trait could explain the absence of some stages in the blood of *M. leprosa*. Both haemogregarines were reported from species of aquatic turtles with partly sympatric distribution (Iverson, 1992; Fritz and Havaš, 2007). Moreover, the developmental stages of *H. bagensis* were also found in the leech *Placobdella costata* (reported also as *Placobdella catenigera* or *Haementeria costata*), the same definitive host and vector for *H. stepanowi* (Brumpt, 1904; Bielecki *et al*. 2012). It would also be interesting to compare the developmental stages in the organs and tissues of the leech vectors, which, however, were not available for this study.

We revealed consistency in the comparison of the 18S rDNA sequences of our isolates, when homologous sequences were identical in haemogregarines from all four turtle host species. Based on our analyses, we assume that a haemogregarine common for *E. orbicularis, M. caspica, M. leprosa* and *M. rivulata* is conspecific with *H. stepanowi*. Nevertheless, we cannot exclude the possibility that

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Table 4. Measurements (μm) of *Haemogregarina* species reported in literature and *H. stepanowi* isolates from four host turtles in this study; na – data not available

Parasite	Host		Trophozoite	Premeront	Meront	Gamont	Reference
H. bagensis	*M. leprosa*	Body	na	8–10 × 2–3	11–20 × 10–14	25–30 × 4–5	Laveran and Pettit (1909)
H. balli	*C. serpentina*	Body	na	na	18·6 (14·5–21) × 7·2 (5–10) (6–8 nuclei)	12·6 (8–14·5) × 5·3 (3–6·5)	Paterson and Desser (1976)
H. stepanowi	*E. orbicularis*	Body	na	na	16 × 6 (4–6 nuclei)	na	Reichenow (1910)
H. stepanowi	*E. orbicularis*	Body	na	na	12·3 × 5·9 (2–4 nuclei)	15,7 × 5	Hahn (1909)
H. stepanowi	*E. orbicularis*	Body	na	10·8 ± 1·0 × 5·1 ± 0·8	12–16 × 6–7 (4–6 nuclei), 15 × 11 (8 nuclei)	32·0 ± 1·9 × 4·2 ± 0·7	Telford (2009)
		Nucleus		4·6 ± 1·5 × 3·8 ± 0·8	na	5·5 ± 0·8 × 3·5 ± 0·5	
H. stepanowi	*E. orbicularis*	Body	10·4 ± 1·7 × 5·5 ± 1·0	9·9 ± 0·9 × 4·3 ± 0·6	13 × 6 (6 nuclei)	32·3 ± 2·1 × 3·2 ± 0·3	This study
		Nucleus	na	4·1 ± 0·3 × 2·3 ± 0·5	na	5·9 ± 0·7 × 3·1 ± 0·5	
		N	10	13	1	30	
H. stepanowi	*M. caspica*	Body	9·6 ± 1·3 × 5·3 ± 0·5	10·9 ± 1·0 × 4·6 ± 0·6	14 × 7	32·5 ± 1·3 × 3·3 ± 0·4	This study
		Nucleus	na	5·4 ± 0·3 × 3·8 ± 1·3	4	5·5 ± 0·7 × 3·9 ± 0·9	
		N	10	19	1	17	
H. stepanowi	*M. leprosa*	Body	12·0 ± 0·0 × 5·0 ± 0·0	11·8 ± 0·8 × 5·4 ± 0·6	na	34·3 ± 1·4 × 3·0 ± 0·2	This study
		Nucleus	na	5·6 ± 0·9 × 3·9 ± 0·7		6·6 ± 1·4 × 2·4 ± 0·5	
		N	2	20		16	
H. stepanowi	*M. rivulata*	Body	9·7 ± 1·0 × 6·0 ± 0·9	11·5 ± 0·5 × 5·5 ± 0·5	13 × 6 (2 nuclei), 19 × 11 (8 nuclei)	32·2 ± 1·9 × 3·6 ± 0·4	This study
		Nucleus	na	5·5 ± 0·8 × 4·3 ± 0·5	na	6·0 ± 1·0 × 4·5 ± 0·8	
		N	14	11	2	29	

Table 5. *P* distances based on total of 1207 positions in the final dataset. The numbers of base differences per site from estimation of net average between groups of sequences are shown. Standard error estimate(s) are shown above the diagonal. The analysis involved 17 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair

	n	*H. balli*	*Hepatozoon* sp. of amphibians and reptiles	*Hepatozoon* of mammals	*Haemogregarina* sp. of African terrapins	*H. stepanowi*
H. balli	1		0·0051	0·0052	0·0025	0·0017
Hepatozoon sp. of amphibians and reptiles	5	0·0450		0·0028	0·0048	0·0051
Hepatozoon sp. of mammals	4	0·0386	0·0151		0·0047	0·0051
Haemogregarina sp. of African terrapins	3	0·0094	0·0408	0·0352		0·0025
H. stepanowi	4	0·0033	0·0440	0·0386	0·0094	

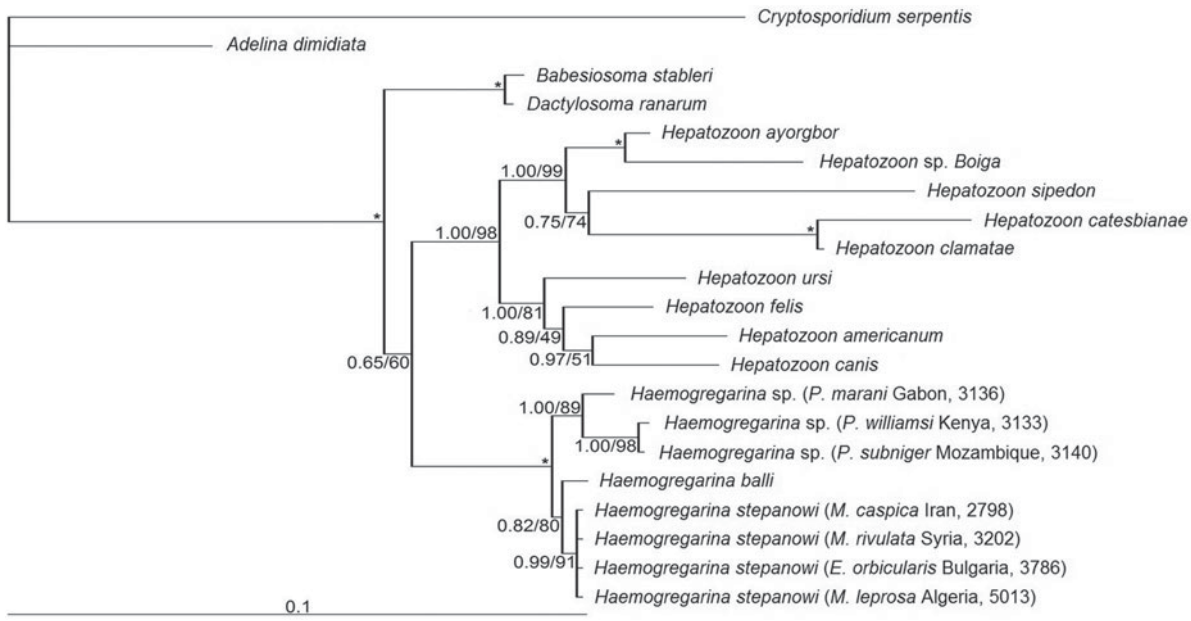

Fig. 2. Bayesian inference phylogenetic tree of 18S rDNA sequences (1207 bases) of haemogregarines. The tree is rooted with *Cryptosporidium serpentis*. Numbers at the nodes show posterior probabilities under BI and bootstrap values for ML, respectively. Maximum posterior probabilities and bootstrap supports 1·0 or 100%, respectively, are marked with asterisk (*).

each turtle species could be parasitized by more than one haemogregarine species that were not detected in this study (e.g. *M. leprosa* by *H. bagensis*). Apicomplexans had already emerged in the Precambrian era and all currently known species are obligatory intracellular parasites (Levine, 1988; Perkins *et al*. 2000). It is believed that their ancestors evolved in invertebrate hosts. Phylogenetic studies suggest that extant species possessing heteroxenous life cycles evolved from monoxenous ancestors parasitizing invertebrates (Barta, 1989; Kopečná *et al*. 2006). A reverse scenario of secondary simplification of complex development is less likely (Landau, 1974). Notably, the vector-transmitted apicomplexans retain their sexual development in the invertebrate host. This trait argues for a low host specificity of haemogregarines regarding their vertebrate host – turtles. We conclude that the presence of this parasite is likely to be strictly linked to the vector and definitive host – the leech.

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