Haemogregarines of freshwater turtles from Southeast Asia with a description of *Haemogregarina sacaliae* sp. n. and a redescription of *Haemogregarina pellegrini* Laveran and Pettit, 1910

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SUMMARY

The uniform morphology of the developmental stages of *Haemogregarina* species and the insufficient information supplied by the simplistic descriptions of previous authors complicates their differential diagnosis and proper species identification. In this study, we detected *Haemogregarina* spp. in 6 out of 22 (27·2%) examined turtles originating from Southeast Asia, *Malayemys subtrijuga* ($n = 4$), *Sacalia quadriocellata* ($n = 1$) and *Platysternon megacephalum* ($n = 1$), and compared them with the available literature data. Microscopic analysis of our isolates distinguished 2 morphological species, *Haemogregarina pellegrini* and one new species, being described in this paper as *Haemogregarina sacaliae* sp. n. Phylogenetic analyses based on 1210 bp long fragment of 18S rDNA sequences placed both haemogregarines firmly within the monophyletic *Haemogregarina* clade. Isolates of *H. pellegrini* from 2 distantly related turtle hosts, *M. subtrijuga* and *P. megacephalum*, were genetically identical. Despite the fact that numerous *Haemogregarina* species of turtles have been described, the incompleteness of the morphological data and relatively low host specificity provides the space for large synonymy within this taxon. Therefore, a complex approach combining microscopic analyses together with molecular-genetic methods should represent the basic standard for all taxonomic studies.

Key words: Haemogregarines, *Haemogregarina*, *Sacalia*, *Platysternon*, *Malayemys*, phylogeny, 18S rDNA.

INTRODUCTION

Representatives of the genus *Haemogregarina* Danilewsky, 1885 are heteroxenous intracellular parasites that infect various species of turtles worldwide (Acholonu, 1974; Siddall and Desser, 1992; Mihalca *et al*. 2002; Telford *et al*. 2009; Davis and Sterrett, 2011; Hossen *et al*. 2013). Their life cycle includes gamogony and sporogony in leeches, and erythrocytic and extra-erythrocytic merogony in chelonian hosts (Desser, 1993; Siddall, 1995). The complete life cycle gives rise to numerous morphologically different life stages, often with temporary occurrence (Desser, 1993; Mihalca *et al*. 2002; Telford, 2009). In the leech host, gamonts are released from the digested blood and monosporoblastic oocysts mature within the intestinal epithelial cells. Sporozoites migrate to anastomosing lacunae of the leech's circulatory system, giving rise to primary merogony. Infectious merozoites enter into the bloodstream of the turtle as the leech feeds. Pre-erythrocytic meronts are formed in the internal organs of the turtle, such as the liver, lungs and spleen; released merozoites enter erythrocytes and become premeronts. After the host cells rupture, the next generation of merozoites infects other erythrocytes where they transform into erythrocytic meronts or gamonts (Reichenow, 1910; Desser, 1993).

Haemogregarines were previously considered to be highly host-specific, and thus each parasite found often led to the description of a new species, yielding 29 named *Haemogregarina* species infecting turtles (Levine, 1988). Further, the majority of them have been described on the basis of the morphological characteristics of a few detected life stages, mostly

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Table 1. Examined turtles

Host species	Collecting site	Number (m/f/juv/nd)
Cuora galbinifrons Bourret, 1939	Vietnam, exact locality unknown	2/0/0/0
Leucocephalon yuwonoi (McCord, Iverson et Boeadi, 1995)	Indonesia, Sulawesi, exact locality unknown	0/0/3/0
Malayemys subtrijuga (Schlegel et Müller, 1845)	Thailand, Isaan province, Surin	4/0/0/6
Malayemys subtrijuga (Schlegel et Müller, 1845)	Thailand, exact locality unknown	0/0/0/2
Malayemys subtrijuga (Schlegel et Müller, 1845)	Vietnam, Go Vap District, Ho Chi Minh City	0/0/0/1
Malayemys subtrijuga (Schlegel et Müller, 1845)	Vietnam, exact locality unknown	0/1/0/0
Sacalia quadriocellata (Siebenrock, 1903)	Vietnam, exact locality unknown	1/0/0/0
Platysternon megacephalum Gray, 1831	China, exact locality unknown	0/1/1/0

m – males, f – females, juv – juveniles, nd – not determined.

the gamonts and meronts found in the erythrocytes of intermediate turtle hosts, offering insufficient information for differential diagnoses. Data on sporogony in the vectors are usually missing; the complete life cycle is known for only a few species (Reichenow, 1910; Paterson and Desser, 1976). Additionally, the host specificity of haemogregarines has also been questioned (Dvořáková et al. 2014).

Twenty three out of 39 *Haemogregarina* species infecting aquatic turtles were identified in Southeast Asia, but most of them are based on century-old-descriptions (e.g. Simond, 1901; Laveran and Mesnil, 1902; Castellani and Willey, 1905; Patton, 1908; Robertson, 1908, 1910; Laveran and Pettit, 1910; Prowazek, 1910; Laveran and Nattan-Larrier, 1912; Mello, 1932*a*, *b*; Misra et al. 1974; Saratchandra, 1981; Ray and Bhattacharjee, 1984; Chai and Chen, 1990; Sinha, 1993). In contrast to all these recorded species and the time which has elapsed since their description, only 2 of them provide more comprehensive data (Robertson, 1910; Ray and Bhattacharjee, 1984). Morphological descriptions of a single life stage accompanied by simple drawings without measurements are attached to some of them, but, more often than not, incomplete development cycles and inadequate information on the morphological characteristics of life stages are presented. The incompleteness of morphological data together with relatively low host specificity provides a space for large synonymy in nomenclature of the genus *Haemogregarina*. A combination of microscopy with widely expanding molecular-genetic methods offers a new powerful tool for the identification of haemogregarines in recent studies, and also for a clarification of the alpha-taxonomy and phylogeny of the genus *Haemogregarina* (Perkins and Keller, 2001; Barta et al. 2012; Dvořáková et al. 2014). Nevertheless, no molecular data have so far been made available for the haemogregarines of Southeast Asia.

In the present study, we examined samples from 5 turtle species originating from Southeast Asia for the presence of *Haemogregarina* species, their morphological comparison, phylogenetic analysis, evaluation of their host specificity, and using the literature data to carry out review of *Haemogregarina* species from the Oriental zoogeographic realm.

MATERIALS AND METHODS

Sampling, microscopy

Twenty-two pet-traded turtles (2 *Cuora galbinifrons*, 3 *Leucocephalon yuwonoi*, 14 *Malayemys subtrijuga*, 2 *Platysternon megacephalum* and one specimen of *Sacalia quadriocellata*) originated from the wildlife in Sulawesi (Indonesia), China, Thailand and Vietnam (Table 1). Unfortunately, the exact capture localities are not known. All the turtles were inspected and sampled during veterinary screening in the Czech Republic. Among 22 sampled turtles, blood smears and blood samples were obtained from 12 turtles, whereas, only blood smears were provided for 10 of the *M. subtrijuga* specimens. Blood was collected from each turtle by puncture of the dorsal coccygeal vein, blood smears were prepared, and the remaining blood was stored in absolute ethanol for the forthcoming molecular-genetic analyses. Smears were fixed in absolute methanol for 5 min, and then stained with Giemsa (diluted 1:10 in distilled water, pH 7) for 15 min. The presence of parasites was observed microscopically using an Olympus BX53 microscope with 1000 × magnification and immersion oil. The intensity of parasitaemia was estimated for each infected turtle by examination of approximately 10^4 erythrocytes (Široký et al. 2005). The maximum length and width were measured for all distinguished developmental stages of the parasites. Further, the LW value (length × width) and the L/W (length/width ratio), maximum length and width (with calculated LW) of nuclei were counted for the gamonts. All measurements are given in μm as a mean followed by SD, with ranges in parentheses. The images were acquired by Quick Photo Camera 3.0 software at 1000 × magnification.

DNA extraction, PCR amplification and sequencing

Blood samples were incubated overnight in lysis buffer with proteinase K before the DNA extraction.

Table 2. List of taxa including GenBank accession numbers of sequences used in this study

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 pellegrini ex *M. subtrijuga*	KM887508	This study
H. pellegrini ex *P. megacephalum*	KM887509	This study
Haemogregarina sacaliae	KM887507	This study
Haemogregarina sp. (*P. marani*, Gabon)	KF257924	Dvořáková *et al*. 2014
Haemogregarina stepanowi (*M. rivulata*, Syria)	KF257927	Dvořáková *et al*. 2014
Hemolivia mariae	KF992711	Kvičerová *et al*. 2014
Hemolivia mauritanica	KF992710	Kvičerová *et al*. 2014
Hemolivia sp.	KF992713	Kvičerová *et al*. 2014
Hemolivia sp.	KF992714	Kvičerová *et al*. 2014
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*. 2006
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

Total genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, eluted in 100 µL of PCR water and stored at −20 °C. The specific primers ER and EF (Kvičerová *et al*. 2008) were used for PCR amplification of approximately 1500 bp long fragment of 18S rDNA of apicomplexans. PCRs were carried out in the total volume of 25 µL using the reaction mixtures and PCR conditions according to Dvořáková *et al*. (2014). Amplicons were visualized by electrophoresis on 1·2% agarose gel using the Midori Green (Elisabeth Pharmacon, Czech Republic). Positive PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) and DNA concentration was then measured with the spectrophotometer Nanodrop ASP-3700 (ACTGene, USA). Sequencing was carried out using an automatic ABI 3730XL DNA analyser (Macrogen Inc., The Netherlands).

Phylogenetic analyses

Obtained sequences were identified by BLAST analysis, edited using the DNASTAR program package (DNASTAR Inc.), and deposited in the NCBI GenBank database (accession numbers KM887507, KM887508 and KM887509). Additional sequences of available *Haemogregarina* species and closely related organisms of the genera *Adelina, Cryptosporidium, Dactylosoma, Hemolivia* and *Hepatozoon* were selected from the GenBank database (NCBI) to reconstruct the phylogeny (Table 2). Alignment was created using BioEdit

(Hall, 1999) with the Clustal W algorithm (Thompson *et al*. 1994). Genetic distances based on the 18S rDNA sequences of different species were generated by the Mega 5.0 (Tamura *et al*. 2011). Methods of Bayesian inference (BI), Maximum likelihood (ML) and Maximum parsimony (MP) were selected to infer the phylogenetic relationships within the related organisms. BI was performed in MrBayes 3.1.2. (Ronquist and Huelsenbeck, 2003) with a GTR+Γ+I model for 10 million generations. Analysis was completed after removing the burn-in of 630 trees. PHYML 2.4.4. (Guindon and Gascuel, 2003) was employed to carry out the ML analysis under the GTR+Γ+I model; bootstrap support was calculated for 1000 replicates. PAUP 4.0b10 (Swofford, 2001) was used to compute the MP, with bootstrap support calculated for 1000 replicates. TreeView 1.6.6 (Page, 1996) was used to visualize the resulting trees, with *Cryptosporidium serpentis* as outgroup.

RESULTS

Morphology of endogenous stages

Blood parasites of the typical morphology of *Haemogregarina* sp. were found by microscopic examination in 6/22 (27·3%) examined turtles belonging to 3 species – *M. subtrijuga* 4/14 (28·6%), *P. megacephalum* 1/2 (50%) and *Sacalia quadriocellata* 1/1 (100%). The highest detected parasitaemia among species was recorded in *S. quadriocellata* (4·58%), followed by *M. subtrijuga* (0·88%) and *P. megacephalum* (0·01%). Morphological analysis of the developmental stages of *Haemogregarina*

parasites found were carried out according to Telford ([2009](#)) and compared with already published morphological data on haemogregarines of turtles from the Oriental zoogeographic region ([Table 3](#)). In our study, one new species and one species identical to *Haemogregarina pellegrini* Laveran and Pettit ([1910](#)), were recorded. Both are described/redescribed as follows:

Description of *Haemogregarina sacaliae* sp. n.

Trophozoites: Smallest life stages ([Fig. 1a](#)) occur individually in erythrocytes, less frequently in pairs, measure $7{\cdot}5 \pm 0{\cdot}9 \times 2{\cdot}7 \pm 0{\cdot}5$ ($6{\cdot}0$–$9{\cdot}0 \times 2{\cdot}0$–$3{\cdot}0$; $n = 20$). Centrally located, distinct, dark purple nucleus covers a large part of the parasite. Cytoplasm is whitish-blue, finely vacuolated.

Meronts: Rarely detected blood stages, containing 3, 4 or 5 nuclei. Meront containing 3 nuclei measures 12×5, meronts with 4 nuclei $12{\cdot}3 \pm 1{\cdot}5 \times 7{\cdot}7 \pm 2{\cdot}1$ ([Fig. 1b](#)), and meront with 5 nuclei 12×5. Cytoplasmatic differentiation into merozoites was visible in one case ([Fig. 1c](#)).

Merozoites: Merozoites found as single, in pairs, or in groups within meronts. Their elongated, worm-like body is sometimes curved ([Fig. 1c](#)). Cytoplasm is vacuolated, usually at both poles. Dark, purple-stained nucleus is disintegrated and centrally situated. Merozoites measure $9{\cdot}7 \pm 0{\cdot}8$ ($9{\cdot}0$–$11{\cdot}0$) $\times 3{\cdot}3 \pm 0{\cdot}5$ ($3{\cdot}0$–$4{\cdot}0$; $n = 30$).

Gamonts: Most abundant life stage, occasionally occurring in pairs, is situated within spacious bean-shaped parasitophorous vacuole occupying approximately half of erythrocyte ([Fig. 1d](#)). Parasite is re-curved in vacuole into 2 branches, but division is clearly visible only in a few gamonts. Blue cytoplasm lacks vacuoles. Dark, purple-stained nucleus is formed by chromatin lumps and located near to one pole. Capsule measure $13{\cdot}2 \pm 0{\cdot}8 \times 6{\cdot}6 \pm 0{\cdot}6$ ($12{\cdot}0$–$15{\cdot}0 \times 6{\cdot}0$–$8{\cdot}0$; $n = 30$), LW is $87{\cdot}4 \pm 10{\cdot}1\ \mu m^2$ (72–112) and L/W ratio is $2{\cdot}0 \pm 0{\cdot}2$ ($1{\cdot}7$–$2{\cdot}5$). Oval nuclei measure $3{\cdot}2 \pm 0{\cdot}4 \times 2{\cdot}7 \pm 0{\cdot}4$ ($3{\cdot}0$–$4{\cdot}0 \times 2{\cdot}0$–$3{\cdot}0$), LW is $8{\cdot}8 \pm 1{\cdot}9\ \mu m^2$ ($6{\cdot}0$–$12{\cdot}0$). Host erythrocyte nucleus is displaced marginally towards edge of cell by all stages of parasites.

Stages in the vector: Unknown
Type host: *Sacalia quadriocellata* (Siebenrock, 1903) (Testudines: Geoemydidae).
Other hosts: Unknown.
Vector: Unknown
Type locality: Vietnam, exact locality unknown.
Other localities: Unknown
Prevalence: A single examined *S. quadriocellata* was parasitized (100%)
Molecular features: 18S rDNA sequence of 1418 bp (GenBank accession number KM887507), GC content 39·1%.

Material deposited: Blood film (marked as VN-34-13) and DNA sample no. 5084 are deposited in the collection of Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

Etymology: The specific epithet *sacaliae* is derived as a genitive of the host generic name *Sacalia*, which is grammatically a feminine.

Remarks. Host species and geography are usually considered in the identification of haemogregarines and their species. No *Haemogregarina* species has been so far reported from turtles of the genus *Sacalia*. Since the host specificity of haemogregarines to their turtle hosts is not strict, all known *Haemogregarina* spp. described from the Oriental zoogeographic region were considered for differential diagnosis with special attention given to the species of turtles of the family Geoemydidae (due to the similar ecology of their representatives). A comparison of the available morphological data on *H. sacaliae* sp. n. shows an overlap in some criteria with *H. nicoriae* Castellani et Willey, [1905](#), *H. mesnili* Simond, [1901](#), *H. stepanowiana* Laveran et Mesnil, [1902](#), *H. pellegrini* Laveran et Pettit, [1910](#), and *H. cuorae* Chai et Chen, [1990](#) ([Table 3](#); Simond, [1901](#); Laveran and Mesnil, [1902](#); Castelani and Willey, [1905](#); Laveran and Pettit, [1910](#); Chai and Chen, [1990](#)). The blood stages of *H. nicoriae, H. mesnili* and *H. stepanowiana* are similar to those of *H. sacaliae*. *H. nicoriae* differs in the significantly smaller dimensions of its gamonts and *H. mesnili* has a larger longitudinally situated nucleus in its gamont life stages. *H. stepanowiana* differs in displaying clearly compound and differentiated branches; its stages do not contain vacuoles and do not form spacious parasitophorous vacuole. Trophozoites and gamonts of *H. pellegrini* as well as merozoites of *H. cuorae* are of a similar size as those of *H. sacaliae*. However, *H. sacaliae* differs primarily in possessing crumbled nuclei and spacious parasitophorous vacuoles in its gamonts and vacuolated merozoites. These traits may be considered as unique, diagnostic features.

Haemogregarines detected in the blood films of 3 individuals of *M. subtrijuga* from Thailand, one *M. subtrijuga* from Vietnam and one *P. megacephalum* from China were identified as *Haemogregarina pellegrini* Laveran et Pettit, [1910](#). Additional data on the developmental stages obtained by the microscopic analysis of blood smears warrant the redescription of *H. pellegrini*.

Redescription of *Haemogregarina pellegrini* Laveran et Pettit, 1910.

Trophozoites: Smallest forms of parasite ([Fig. 1e](#)), elongated and slightly curved with bounded nucleus consisting of accumulated mass of chromatin at mid-body; several vacuoles and dark purple granules are sometimes found in cytoplasm. Size is $7{\cdot}8 \pm 0{\cdot}6 \times 3{\cdot}2 \pm 0{\cdot}5$ ($7{\cdot}0$–$9{\cdot}0 \times 2{\cdot}0$–$4{\cdot}0$; $n = 30$).

Table 3. Review of basic morphology of *Haemogregarina* species from turtles of East and South Asia

Parasite	Intermediate host	Origin		Trophozoite	Premeront	Merozoite	Meront	Gamont	Reference
H. bongaonensis	*Nilssonia gangetica*	India	Body	4·5–13·3 × 2·8–7·8	na	6·5 × 3	7–14·5 × 5·5–9·8	6·5–13·6 × 1·3–4·4	Sinha (1993)
H. botuliformis	*Mauremys reevesi*	China	Body	25·36–25·84 × 7·08–7·16	na	na	17·12–17·46 × 7·02–7·18 (4–8 nuclei)	16·24–16·40 × 6·10–6·14	Chai and Chen (1990)
H. chinemydis	*M. reevesi*	China	Body	25·24–25·76 × 7·08–7·28	na	na	17·12–17·46 × 6·50–6·65 (10–14 nuclei)	17·26–19·46 × 4·02–5·12 5·08–13·32 × 3·02–4·10	Chai and Chen (1990)
			Nucleus	na			na		
H. choudhuryi	*Lissemys p. punctata*	India	Body	na	na	5·7 × 1·0	10·0 × 5·5	8·0 × 2·5	Ray and Bhattacharjee (1984)
H. clemmydis	*Mauremys japonica*	Japan	Body	na	10 × 4	na	na	na	Prowazek (1910)
H. cuorae	*Cuora flavomarginata*	China	Body	na	na	8·12–8·20 × 3·02–3·08	13·14–13·32 × 6·08–6·14	16·14–18·46 × 2·00–3·02 11·14– 11·28 × 2·46–3·02	Chai and Chen (1990)
			Nucleus	na		na	na		
H. emydae	*Pelodiscus sinensis*	Japan	Body	na	na	na	na	8 × 4	Prowazek (1910)
H. galeata	*P. sinensis*	China	Body	16·12–16·64 × 7·06–7·18	na	na	15·12–15·46 × 6·02–6·18	11·12–13·32 × 5·04–5·12	Chai and Chen (1990)
H. ganapatii	*L. p. granosa*	India	Body	7 × 3	8 × 3·8	na	9·5–11·5 × 5·5–6·5 (4–8 nuclei)	6–11·5 × 3–4·5	Saratchandra (1981)
H. gangetica	*N. gangetica*	India	Body	na	na	na	na	9·16–9·6 × 3·2–4·8	Misra *et al.* (1974); Misra (1976)
H. hubeiensis	*P. sinensis*	China	Body	na	na	7·10–11·24 × 2·00–4·10	na	na	Chai and Chen (1990)
H. malabarica	*L. punctata*	India	Body	na	lenght 8–12	na	na	length 17–22	Mello (1932a, 1932b)
H. nicoriae	*Melanochelys trijuga*	Sri Lanca	Body	na	na	na	na	length 10	Castellani and Willey (1905)
H. pellegrini	*M. subtrijuga*	Southeast Asia	Body	6–7 × 2–3	7–11 × 2·5–4	na	na	11–15 × 4–6	Laveran and Pettit (1910)
H. rara	*M. reevesi*	East Asia	Body	na	na	na	na	15 × 2–3	Laveran and Mesnil (1902)
H. sinensis	*P. sinensis*	China	Body	na	na	8·02–8·20 × 2·00–2·06	7·02–14·26 × 2·00–8·20	10·92–12·08 × 4·02–5·12	Chai and Chen (1990)
H. stepanoviana	*M. reevesi*	East Asia	Body	na	na	na	(2–12 nuclei) na	18–20 × 5	Laveran and Mesnil (1902)
H. testudinis	*Manouria emys*	Indo–Malaysia	Body	na	na	na	15–20 × 12–14	11–13 × 5–6	Laveran and Nattan-Larrier (1912)
H. vittatae	*L. p. vittata*	Sri Lanca	Body	na	na	na	na	lenght 22–26	Robertson (1908)

Species	Hosts	Locality	Stage						Reference
H. pellegrini	*Malayemys subtrijuga, P. megacephalum*	Thailand, Vietnam, China	Body	7·8 ± 0·6 × 3·2 ± 0·5	9·3 ± 1·1 × 4·7 ± 0·7		11·3 ± 1·0 × 6·3 ± 1·1 (2–4 nuclei)	15·2 ± 2·0 × 6·1 ± 1·3 × 5·6 ± 0·9 × 3·4 ± 0·6	This study
			Nucleus	na	na		na	na	
			N	30	30		7	60	
H. sacaliae	*Sacalia quadriocellata*	Vietnam	Body	7·5 ± 0·9 × 2·7 ± 0·5	na	9·7 ± 0·8 × 3·3 ± 0·5	12·3 ± 1·2 × 7·0 ± 2·0 (3–5 nuclei)	13·2 ± 0·8 × 6·6 ± 0·6 × 3·2 ± 0·4 × 2·7 ± 0·4	This study
			Nucleus	na		na	na	na	
			N	20	30	30	8	30	

na – data not available. Dimensions of individual developmental stages of *H. billeti*, *H. laverani*, *H. mesnili* (Simond, 1901) and *H. xavieri* (Mello, 1932b) were not specified in the literature. All the measurements are provided in μm.

Nucleus of infected erythrocyte is often displaced to polar position.

Premeronts: Oval sometimes elongated stages with vacuolated cytoplasm and granules (Fig. 1f). They measure $9·3 ± 1·1 × 4·7 ± 0·7$ ($8·0$–$12·0 × 4·0$–$6·0$; $n = 30$). Nuclei consist of mass of chromatin located usually in central position.

Early meronts: Elongated stages measuring $12·1 ± 0·7 × 5·7 ± 0·6$ ($11·0$–$13·0 × 5·0$–$7·0$; $n = 15$) occur in erythrocytes individually. Inclusions of chromatin situated mostly along periphery, sometimes scattered within parasite in stained deep blue cytoplasm (Fig. 1g).

Meronts: Binucleate and multinucleate meronts found in bloodstream, elongated, slightly recurved. Immature meronts with 2 small nuclei measured $10·8 ± 1·0 × 6·0 ± 1·4 \mu m$ ($10·0$–$12·0 × 4·0$–$7·0$; $n = 4$), 2 meronts with 3 nuclei (Fig. 1h) $12 × 6$ and $12 × 7$, respectively, and single meront with 4 nuclei measure $12 × 7$.

Gamonts: Two distinguishable forms are detected – microgamonts (Fig. 1i) and macrogamonts (Fig. 1j,k). Estimated number of macro- and microgamonts found in individual smears is similar. Macrogamonts represent largest encapsulated life stage with significantly more stained cytoplasm compared with microgamonts. Formation of 2 branches is not clearly visible. Both forms elongated, slightly recurved (cucumber-shaped), usually place longitudinally along full length of host's erythrocytes long axis. Nucleus of young gamonts is localized significantly closer to 'anterior' wider end ('head') than to 'posterior' sharp end ('tail'). Posterior end is sometimes bent.

Mostly compact and basophilic nucleus of oval, circular, or sometimes banded shape occupy full width of gamont. It is situated eccentrically, in macrogamonts, also sometimes centrally. Whitish-purple polar cap fills one-third of interior space on opposite pole to nucleus placement. Basophilic granules are sometimes visible in microgamonts.

Capsule of macrogamont averages $16·9 ± 1·1 × 7·2 ± 0·8$ ($15·0$–$19·0 × 6·0$–$9·0$; $n = 30$), with LW $119·5 ± 17·7 \mu m^2$ (96–152) and L/W ratio $2·36 ± 0·26$ ($1·77$–$3·00$). Their nuclei are $6·2 ± 0·7 × 3·6 ± 0·6$ ($5·0$–$7·0 × 3·0$–$5·0$) with LW $22·3 ± 4·5 \mu m^2$ ($15·0$–$30·0$). Microgamont capsules measure $13·5 ± 1·0 × 5·0 ± 0·7$ ($12·0$–$15·0 × 4·0$–$6·0$; $n = 30$), with LW $66·9 ± 8·4 \mu m^2$ (52–90) and L/W $2·79 ± 0·52$ ($2·00$–$3·75$). Dimensions of nuclei are $5·0 ± 0·8 × 3·3 ± 0·6$ ($4·0$–$7·0 × 2·0$–$4·0$), with LW $16·6 ± 4·04 \mu m^2$ ($10·0$–$24·0$). Host erythrocyte nuclei are displaced towards periphery of cell and strongly compressed.

Type host: *Malayemys subtrijuga* (Schlegel et Müller, 1845) (Testudines: Geoemydidae).

Other hosts: *Platysternon megacephalum* Gray, 1831 (Testudines: Platysternidae).

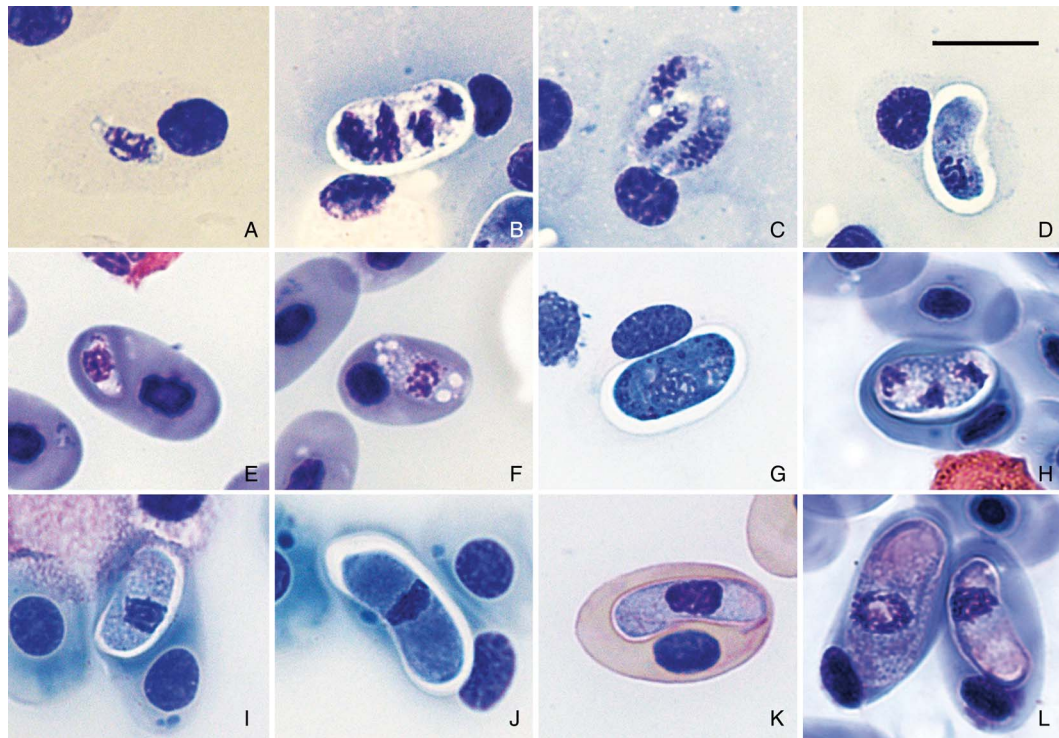Type locality: Southeast Asia, exact locality unknown (Laveran and Pettit, 1910).

Fig. 1. Morphology of *H. sacaliae* n. sp. (a–d) and *H. pellegrini* (e-l). Life-stages of *H. sacaliae* detected in the present study – trophozoite (a), meront (b), differentiated merozoites (c), and gamont (d). Life-stages of *H. pellegrini* – trophozoite (e), premeront (f), early meront (g), meront (h), microgamont (i), macrogamont from *M. subtrijuga* for comparison (j) and from *P. megacephalum* (k), both micro- and macrogamont from *M. subtrijuga* (l). All figures are in the same scale; scale bar = 10 µm.

Other localities (this study): Surin, Isaan province, Thailand. China and Vietnam – for both, exact locality unknown.

Prevalence: 4 of 14 (28·6%) *M. subtrijuga* and 1 of 2 (50%) *P. megacephalum* parasitized.

Molecular features: 18S rDNA sequence of 1423 bp (GenBank accession number KM887508), GC content 39·4%.

Material deposited: Blood films (marked as TH-1-06, TH-2-06, TH-3-06, VN-37-13 and CHI-33-13) and DNA samples no. 5093 and 5083 are deposited in the collection of Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

Remarks. *H. pellegrini* was described from a single specimen of *M. subtrijuga* by Laveran and Pettit in 1910. They described it as croissant-shaped parasite inside a capsule with rounded extremities or one tapered end. Its granulated cytoplasm contained an undifferentiated nucleus. The free parasite measured 10–14 × 2–3 µm. The authors identified small, medium and large forms. The small forms were usually spherical or oval in shape, measuring 6–7 × 2–3 µm, their nuclei consisted of more or less compact grains of chromatin. The most common forms – medium forms – were cylindrical, 7–11 × 2·5–4 µm, usually curved and with rounded ends, sometimes one slightly tapering with a pale-blue, vacuolated cytoplasm. Chromophilic granules were sometimes present; the nucleus was identical to nucleus of the small forms. Large forms measuring 11–15 × 4–6 µm were situated in the main axis of erythrocytes, rarely recurved with a nucleus consisting of chromatin particles often located in the middle of the parasite. The cytoplasm contained numerous chromophilic granules. The erythrocytic nucleus was displaced to the edge of the cell, and slightly enlarged and deformed by the surrounding pressure of the parasite. We have distinguished life stages according to Telford (2009), where we have considered the small forms to be trophozoites, the medium forms premeronts, and the large forms gamonts. Additionally, we found that trophozoites may contain dark purple granules. Premeronts possessed a significantly vacuolated cytoplasm with granules. In terms of gamonts (the large forms), we have discerned macro- and microgamonts (Fig. 1l); we assumed that Laveran and Pettit (1910) classified both macro- and microgamonts as a single form. Despite their descriptions being very similar, their nuclei may be located centrally and the stages may include basophilic granules. All individual developmental stages are comparable in their sizes.

Molecular characteristics and phylogeny

In our molecular analyses, 18S rDNA sequences of 3 samples were included. The obtained sequences of

Table 4. *P-distances based on total of 1210 positions in the final dataset*

	n	Hemolivia sp.	Hepatozoon sp. of amphibians and reptiles	Hepatozoon sp. of mammals	Haemogregarina sp. of Pelusios marani	Haemogregarina pellegrini	Haemogregarina sacaliae	Haemogregarina balli, H. stepanowi
Hemolivia sp.	4	0·011						
Hepatozoon sp. of amphibians and reptiles	5	0·021	0·065					
Hepatozoon sp. of mammals	4	0·013	0·016	0·035				
Haemogregarina sp. of *Pelusios marani*	1	0·045	0·050	0·043	n/c			
Haemogregarina pellegrini	2	0·042	0·045	0·038	0·015	0·000		
H. sacaliae	1	0·041	0·044	0·038	0·011	0·009	n/c	
Haemogregarina balli, *H. stepanowi*	2	0·040	0·044	0·037	0·009	0·006	0·006	0·003

The numbers of base differences per site from an estimation of net average within each group and between groups of sequences are shown. All positions containing gaps and missing data were eliminated. The presence of n/c in the results denote cases in which only one sequence was contained.

the lengths 1411 bp, 1418 bp and 1423 bp were used to calculate *p*-distances (see Table 4). Phylogenies were based on the alignment of 1210 bp with a total of 19 sequences. All analyses (BI, ML and MP) provided identical topologies, however, with different node supports (Fig. 2). A monophyletic cluster was formed by the 3 main branches: (1) the single *Dactylosoma ranarum*; (2) the well-supported *Hemolivia-Hepatozoon* clade comprising the *Hepatozoon* species of mammals, *Hepatozoon* spp. of amphibians and reptiles and *Hemolivia* spp. of turtles and skink; (3) the clade consisting of *Haemogregarina* species. A haemogregarine from *S. quadriocellata* represented a sister taxon to *Haemogregarina* from the African hinged terrapin, *Pelusios marani*, from Gabon. Their closest relatives were *H. balli* and *H. stepanowi*. Haemogregarines from *M. subtrijuga* and *P. megacephalum* were genetically identical, thus conspecific, and constituted a sister taxon to all other *Haemogregarina* species included in the analysis.

DISCUSSION

To date, a number of *Haemogregarina* species has been described from numerous turtle hosts and broad geographic areas. Their differentiation based only on morphological characteristics is considerably limited (Telford *et al.* 2009). Most frequently observed developmental stages often possess a uniform morphology, which complicates the certainty of assignment of found haemogregarines to the previously described taxa. Stages from the definitive hosts, which are helpful in differential diagnosis, are known for only 2 *Haemogregarina* species described from studied region – *H. nicoriae* and *H. choudhuryi* (Ray and Bhattacharjee, 1984; Robertson, 1910). The conspecificity of any 2 given haemogregarines can only be hypothesized when studied isolates originate from the same area or from the same or related turtle hosts, and at that, with a degree of caution. Nevertheless, it has been proved repeatedly that a single *Haemogregarina* species can infect several turtle species from different families (Paterson and Desser, 1976; Siddall and Desser, 1992; Telford *et al.* 2009; Dvořáková *et al.* 2014). Although the employment of molecular-genetic tools is helpful in ascertaining the taxonomy of recently examined or described species, these methods are inapplicable to earlier descriptions, where sequence data are missing. In such cases, microscopy remains the only useful method, since the blood stages of the same *Haemogregarina* species evince only slight morphological differences when infecting the hosts of different families (Dvořáková *et al.* 2014). Similarly, the geographic distribution of one haemogregarine species may span thousands of kilometres, and as was suggested
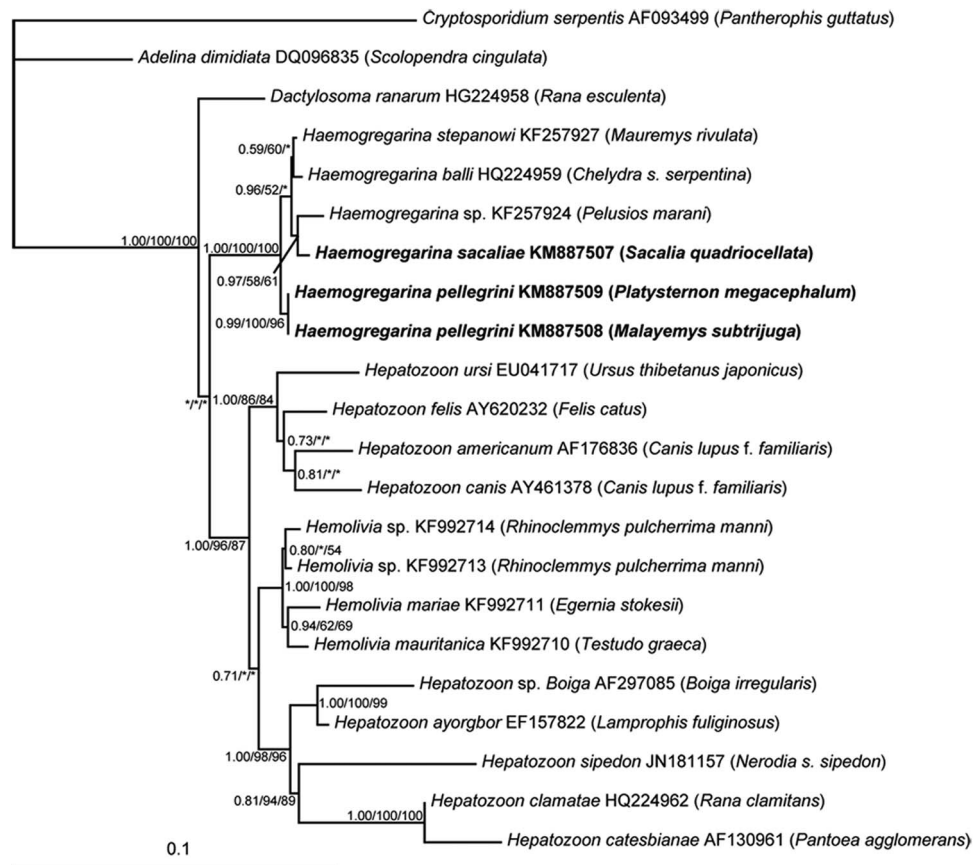
Fig. 2. Phylogenetic tree of *Haemogregarina* sp. inferred from partial 18S rDNA sequences. Numbers at the nodes show posterior probabilities under BI/ bootstrap values for ML/MP higher than 0·50 or 50%, respectively. Posterior probabilities and bootstrap that supports lower than 0·50 or 50% are marked with asterisk (*). Taxa for which new sequences were obtained in this study are printed in bold.

in the case of *H. stepanowi*, the occurrence of haemogregarines probably depends on the range of its definitive host – the leech (Reichenow, 1910; Bielecki *et al.* 2012; Dvořáková *et al.* 2014). Hence, the risk of infection probably exists anywhere the leech vector and appropriate turtle host occur together.

The vector is not necessarily always only a single leech species; for example, *H. balli* is transmitted by 2 related leeches – *Placobdella parasitica* and *P. ornata* (Paterson and Desser, 1976). The prevalence of leeches on their turtle hosts varies depending on the microhabitat predominantly occupied by the turtle. Aerial-basking reduces leech loads through desiccation and predation (McAuliffe, 1977), while bottom-dwelling close to the substrate where leeches reside causes heavier infestation (McCoy *et al.* 2007). Accordingly, we suggest that a single haemogregarine species may parasitize a group of turtle species occupying the same or very similar ecological niches.

Our findings were compared with available morphological data of formerly described species from the studied region, although the published descriptions of parasites were very simplistic in some cases; e.g. *H. clemmydis* and *H. emydae* (Prowazek,

1910). We confirmed that the host specificity of haemogregarines is not strict, since we found life stages of a single *Haemogregarina* sp. in the bloodstream of 2 turtle species belonging to different families, but occurring in similar semiaquatic habitats. In general appearance, a haemogregarine infecting *M. subtrijuga* and *P. megacephalum* was identical and comparable in size to *H. pellegrini* recorded by Laveran and Pettit (1910) also from *M. subtrijuga*. The only authors reporting a survey for haemogregarines from *P. megacephalum* were Chai and Chen (1990), who examined 2 individuals, but did not detect any haemogregarines. Compared to Laveran and Pettit (1910), we were able to distinguish macrogamonts and microgamonts in our slides. Discovered haemogregarine from *S. quadriocellata* – *H. sacaliae* differs from previously described species of this region in a combination of vacuolated merozoites, crumbled nucleus and the spacious parasitophorous vacuole of gamonts. However, the parasite was detected in a single turtle, and we cannot exclude that crumbled nucleus of gamonts may represent only their younger forms. 18S rDNA sequences of *H. pellegrini* from both tortoise hosts, *M. subtrijuga* and *P. megacephalum*, were identical. Phylogenetic analyses based on 18S rDNA placed

both *H. pellegrini* and *H. sacaliae* firmly within the *Haemogregarina* clade (Fig. 2). The availability of sequences of a single gene from a few *Haemogregarina* species did not allow for any co-evolutionary or phylogeographic analyses.

Our study provides the first *Haemogregarina* sequences from the region of Southeast Asia. It is surprising that despite the large number of already described *Haemogregarina* species from Asian turtles, none of them has been recorded repeatedly. Descriptions of new species are published continuously instead of trials to confirm the presence of already known species, to specify their host spectra, and to characterize their geographic ranges. Under such circumstances, a considerable synonymy of haemogregarines in this region is very probable (Siddall, 1995). Hence, the applications of molecular-genetic characteristics (barcodes) together with rigorous morphological analysis are necessary prerequisites for avoiding further taxonomic inflation in this group.

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