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ORIGINAL PAPER

New species of haemosporidian parasites (Haemosporida) from African rainforest birds, with remarks on their classification

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Abstract *Plasmodium* (*Novyella*) *megaglobularis* n. sp. was recorded in the olive sunbird *Cyanomitra olivacea*, and *Plasmodium* (*Novyella*) *globularis* n. sp. and *Haemoproteus* (*Parahaemoproteus*) *vacuolatus* n. sp. were found in the yellow-whiskered greenbul *Andropadus latirostris* in rainforests of Ghana and Cameroon. These parasites are described based on the morphology of their blood stages and a segment of the mitochondrial cytochrome *b* gene, which can be used for molecular identification and diagnosis of these species. Illustrations of blood stages of new species are given, and phylogenetic analysis identifies deoxyribonucleic acid (DNA) lineages closely related to these parasites. Traditional taxonomy of avian pigment-forming haemosporidians of the families Plasmodiidae and Haemoproteidae is discussed based on the recent molecular phylogenies of these parasites. We conclude that further work to increase the number of precise linkages between haemosporidian DNA sequences and their corresponding morphospecies is needed before revising the current classification of haemosporidians. This study emphasises the value of both the polymerase chain reaction and microscopy in the identification of avian haemosporidian parasites.

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Introduction

As part of a comprehensive ongoing study on the effects of deforestation on the prevalence of blood pathogens in African rainforest birds, large numbers of blood samples from African rainforest birds were collected in Ghana and Cameroon in 2005–2007. Several previously undescribed species of avian blood parasites were discovered during this study. Three of these species belonging to the families Plasmodiidae and Haemoproteidae (the order Haemosporida) were recorded in two species of passeriform birds, the yellow-whiskered greenbul *Andropadus latirostris* and the olive sunbird *Cyanomitra olivacea*. Both bird species are widespread in African rainforests (Borrow and Demey 2005) and serve as convenient model organisms in ecological and evolutionary biology studies for blood parasite infections (Sehgal et al. 2001, 2005). Three new species of parasites are described here using data on the morphology of their blood stages and sequences of the mitochondrial cytochrome *b* (cyt *b*) gene.

The aims of this study were (1) to provide detailed morphological descriptions of new species, (2) to determine mitochondrial cyt *b* gene sequences, which can be used for molecular identification and diagnosis of these parasites, (3) to compare genetic distances and phylogenetic relationships of new species with positively identified species belonging to the same genera and sub-genera and (4) to discuss classification of new species based on the present molecular phylogenies of avian haemosporidians.

Materials and methods

Collection of blood samples

The samples were collected in Ghana and Cameroon at 25 sites in both rainy and dry seasons during 2005–2007. In all, samples from 449 adult olive sunbirds and 187 adult yellow-whiskered greenbuls were used in this study. The birds were caught with mist nets and banded. Blood samples were collected from the brachial vein, and birds were released with none of the individual being re-captured.

Three blood films were prepared from each bird. Blood films were air-dried within 5–10 s after their preparation. In humid environments, we used a battery-operated fan to aid in the drying of the blood films. Slides were fixed in absolute methanol in the field and then stained with Giemsa in the laboratory. Blood films were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000). Detailed protocols of fixation, staining and microscopic examination of blood films were described by Valkiūnas et al. (2008c). Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light (i.e. <0.1%), as recommended by Godfrey et al. (1987). To ensure absence of simultaneous infections with haemosporidian parasites in the type material of new species, the entire blood films from hapantotype and parahapantotype series were examined microscopically at low magnification.

Approximately 50 μl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were fixed in lysis buffer (Sehgal et al. 2001); they were held at ambient temperature in the field and later at −20°C in the laboratory.

Morphological analysis

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine slides, prepare illustrations and to take measurements. The morphometric features studied (Table 1) were those defined by Valkiūnas (2005).

Morphology of the parasites was compared with type and voucher specimens of *Plasmodium* (*Novyella*) *rouxi* from its type vertebrate host, the Spanish sparrow *Passer hispaniolensis* (a passage to canary, blood slide accession no. 650), and from its common host, the house sparrow *Passer domesticus* (accession nos. 648, 649), in the Garnham Collection at the Natural History Museum, London, and with type material of *Plasmodium* (*Novyella*) *bertii* from its type hosts, the grey-necked wood rail *Aramides cajanea* (accession no. 960). Student's *t* test for independent samples was used to determine statistical significance between mean linear parameters. A *P* value of 0.05 or less was considered significant.

DNA extraction, PCR amplification and sequencing

Deoxyribonucleic acid (DNA) was extracted from whole blood following a DNeasy kit protocol (Qiagen, Valencia, CA, USA). Success of each DNA extraction was verified with primers that amplify the gene encoding the brain-derived neurotrophic factor (Sehgal and Lovette 2003).

For *Plasmodium* and *Haemoproteus* spp. detection, we used a polymerase chain reaction (PCR) method that amplifies a fragment of the cyt *b* region of the mitochondrial DNA with primers L15183: 5′-GTG CAA CYG TTA TTA CTA ATT TAT A-3′ and H15730: 5′-CAT CCA ATC CAT AAT AAA GCA T-3′ (Fallon et al. 2003; Szymanski and Lovette 2005). The cycling profile consisted of an initial denaturing at 94°C for 3 min, followed by 35 cycles of 94°C for 50 s, 53°C annealing for 50 s and 72°C extension for 60 s and then a final extension at 72°C for 5 min. Positive and negative controls were used. The positive controls were from infected birds, as determined by microscopic examination of blood films, and the negative controls were purified water in place of DNA template or samples that were consistently void of parasites, as confirmed both by microscopic examination and PCR. We also used an additional nested PCR to amplify a fragment of the cyt *b* of new species of *Plasmodium* sp. and *Haemoproteus* sp. from infected birds with the primers HAEMF/HAEMR2–HAEMNF/HAEMNR2 following the protocol of Waldenström et al. (2004).

PCR products were purified using ExoSap following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA). All lineages were identified by sequencing the PCR products (BigDye® version 1.1 sequencing kit, Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100™ automated sequencer (Applied Biosystems).

We used both PCR methods to compare the lineages of new species with sequences of parasites belonging to the genera *Plasmodium* and *Haemoproteus* deposited in GenBank. Sequences obtained with the nested PCR protocol were used for construction of the phylogenetic tree. The GenBank sequences used were carefully selected to correspond to positive morphological identifications (see Valkiūnas et al. 2008d).

Phylogenetic analysis

We used 13 mitochondrial cyt *b* sequences of avian *Plasmodium* spp. and 14 of *Haemoproteus* spp. from our survey and GenBank. The GenBank accession numbers of

Table 1 Morphometry of host cells and mature blood stages of new species of haemosporidian (Haemosporida) parasites from African rainforest birds (n=21)

Feature	Measurements (μm)[a]		
	Plasmodium globularis Lineage pANLA1	*Plasmodium megaglobularis* Lineage pCYOL1	*Haemoproteus vacuolatus* Lineage hANLA1
Uninfected erythrocyte			
Length	10.2–12.1 (11.3±0.5)	9.4–12.0 (10.5±0.7)	10.3–12.5 (11.3±0.6)
Width	5.8–7.2 (6.5±0.4)	5.4–7.0 (6.2±0.4)	5.3–8.0 (6.4±0.6)
Area	51.9–68.3 (60.1±3.9)	54.8–70.4 (62.2±4.8)	48.2–68.6 (59.2±6.5)
Uninfected erythrocyte nucleus			
Length	4.9–6.0 (5.5±0.3)	3.7–6.0 (5.1±0.6)	4.8–6.0 (5.4±0.3)
Width	2.2–2.8 (2.5±0.2)	1.5–3.2 (2.3±0.4)	2.3–3.0 (2.7±0.2)
Area	11.2–15.0 (12.4±0.9)	10.0–13.7 (11.8±1.0)	10.8–15.8 (12.8±1.2)
Macrogametocyte			
Infected erythrocyte			
Length	10.8–13.1 (12.0±0.6)	9.4–12.9 (12.0±0.8)	12.0–14.7 (13.2±0.6)
Width	6.2–7.6 (6.8±0.3)	5.2–7.1 (6.5±0.5)	5.9–8.1 (7.1±0.5)
Area	59.7–77.8 (66.9±5.3)	50.8–69.0 (60.8±5.2)	63.9–88.1 (77.6±6.0)
Infected erythrocyte nucleus			
Length	4.8–6.1 (5.5±0.3)	4.4–6.4 (5.3±0.5)	5.1–6.0 (5.5±0.2)
Width	2.2–2.7 (2.4±0.1)	1.8–3.2 (2.3±0.4)	2.0–3.0 (2.5±0.3)
Area	9.8–14.0 (12.2±1.0)	9.8–15.6 (11.4±1.4)	9.3–15.8 (12.6±1.5)
Gametocyte			
Length	8.1–10.7 (9.3±0.6)	10.6–15.3 (12.7±1.2)	10.9–14.5 (12.5±1.1)
Width	1.0–2.0 (1.4±0.3)	1.6–3.9 (2.4±0.5)	2.1–3.0 (2.5±0.3)
Area	9.5–16.3 (13.1±1.9)	24.2–41.5 (31.3±4.7)	24.0–42.3 (33.7±5.1)
Gametocyte nucleus			
Length	–	–	1.7–3.8 (2.6±0.5)
Width	–	–	0.5–1.9 (1.2±0.4)
Area	0.8–1.9 (1.3±0.3)	1.2–2.9 (1.9±0.5)	1.6–4.5 (2.7±0.6)
Pigment granules	2.0–6.0 (4.7±1.3)	6.0–13.0 (9.6±2.1)	15.0–27.0 (20.3±4.1)
NDR	0.8–1.2 (1.0±0.1)	0.4–1.0 (0.6±0.2)	0.6–1.0 (0.8±0.1)
Microgametocyte			
Infected erythrocyte			
Length	10.9–13.6 (11.7±0.6)	10.8–13.0 (12.2±0.6)	11.4–13.8 (12.6±0.6)
Width	6.0–7.6 (6.8±0.5)	5.8–6.9 (6.4±0.3)	6.8–8.2 (7.5±0.4)
Area	56.5–73.4 (64.1±5.4)	54.4–73.7 (63.7±5.0)	67.1–86.4 (76.4±5.0)
Infected erythrocyte nucleus			
Length	4.9–6.2 (5.6±0.3)	4.8–6.1 (5.4±0.3)	4.7–5.8 (5.3±0.3)
Width	2.1–3.0 (2.5±0.2)	1.9–2.9 (2.3±0.3)	2.1–3.1 (2.4±0.2)
Area	10.7–15.2 (12.2±1.1)	9.6–14.2 (11.4±1.2)	9.3–13.2 (11.2±1.0)
Gametocyte			
Length	8.3–11.9 (9.6±0.8)	10.1–13.8 (11.9±1.0)	11.0–13.9 (12.4±1.0)
Width	1.0–2.1 (1.7±0.3)	1.9–3.3 (2.5±0.4)	2.2–3.4 (2.8±0.3)
Area	12.4–24.7 (16.6±2.9)	26.5–36.7 (31.5±4.3)	30.2–48.0 (36.6±4.5)
Gametocyte nucleus			
Length	–	–	3.5–6.8 (4.9±0.8)
Width	–	–	1.6–2.7 (2.2±0.3)
Area	2.0–6.4 (4.3±1.0)	5.1–9.0 (6.6±1.1)	3.9–11.6 (8.3±1.9)
Pigment granules	3.0–8.0 (5.2±1.2)	6.0–13.0 (9.5±2.0)	17.0–29.0 (22.3±3.3)
NDR	0.7–1.2 (0.9±0.1)	0.3–0.8 (0.6±0.1)	0.6–0.9 (0.7±0.1)
Meront			
Length	3.0–4.7 (3.6±0.4)	3.1–5.9 (4.1±0.8)	–
Width	1.1–2.0 (1.5±0.2)	1.2–2.8 (2.0±0.5)	–
Area	3.9–6.0 (4.6±0.7)	5.6–8.1 (7.0±0.7)	–
Area of globule	0.3–0.7 (0.4±0.1)	1.0–2.3 (1.4±0.3)	–
No. of pigment granules	1–2 (1.2±0.4)	1–3 (1.7±0.7)	–
No. of merozoites	4	4	–

[a] Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.
NDR Nucleus displacement ratio according to Bennett and Campbell (1972)

these sequences are given in Fig. 5. The sequences were aligned using Sequencer 4.8 (GeneCodes, Ann Arbor, MI, USA). All individual sequences were grouped into a consensus that was 468 bp long with two lineages of *Leucocytozoon schoutedeni* used as outgroups (Fig. 5).

The phylogenetic tree was constructed using Bayesian phylogenetics as implemented in mrBayes version 3.1 (Ronquist and Huelsenbeck 2003) after finding an appropriate model of sequence evolution using the software mrModeltest (Nylander 2004). A general time-reversible model including invariable sites (GTR+I) was used. The Bayesian phylogeny was obtained using one cold and two hot Monte Carlo Markov chains, which were sampled every 200 generations over 20 million generations; 100,000 trees were generated. Of these trees, 25% were discarded as burn-in material. The remaining 75,000 trees were used to construct a majority consensus tree.

The sequence divergence between the different lineages (Tables 2 and 3) was calculated with the use of a Jukes–Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA 3.1 (Kumar et al. 2004).

Results

Description of parasites

Plasmodium (Novyella) globularis n. sp.

Type host The host is the yellow-whiskered greenbul *A. latirostris* (Passeriformes, Picnonotidae).

DNA sequences The DNA sequence is mitochondrial cyt *b* lineage pANLA1 (468 bp, GenBank accession no. EU770151).

Additional hosts Additional hosts are unknown.

Type locality Its locality is Agumatsa, Ghana (07°01.758′ N, 00°33.490′ E, 269 m above sea level).

Prevalence Overall prevalence in the yellow-whiskered greenbul was 10 of 187 (5.3%). In the type locality, the prevalence was 5 of 45 (11.1%). According to microscopic examination and PCR diagnostics, two of all recorded infections of *P. globularis* were mixed with *Haemoproteus vacuolatus*. The samples with single infections were used for the description of *P. globularis*.

Distribution This morphospecies and its lineage pANLA1 have been recorded only in Ghana so far. Geographical distribution is unclear. This parasite has not been found in European migrants subsequent to their arrival from African wintering grounds. It is probable that this lineage is host-restricted and is transmitted in Africa, where it might be patchy in distribution.

Site of infection The sites of infection are mature erythrocytes; no other data were available.

Type specimens The hapantotype (accession number 41645 NS, intensity of parasitaemia is 0.1%, *A. latirostris*, Agumatsa, Ghana, collected by G. Valkiūnas, 7 July 2007) is deposited in the Institute of Ecology, Vilnius

Table 2 The sequence divergence (in percentage) between mitochondrial cytochrome *b* lineages of positively identified species of *Haemoproteus*

Species [a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 *H. columbae*	0													
2 *H. pallidus*	15.4	0												
3 *H. pallidus*	15.1	0.2	0											
4 ***H. vacuolatus***	15.9	1.9	1.7	0										
5 *H. minutus*	15.6	0.6	0.4	1.7	0									
6 *H. balmorali*	16.5	5.5	5.3	5.5	5.8	0								
7 *H. balmorali*	16.7	4.9	4.6	5.3	5.1	3.1	0							
8 *H. balmorali*	15.9	5.3	5.1	5.3	5.5	3.1	2.4	0						
9 *H. belopolskyi*	14.3	5.5	5.3	5.8	5.8	7.2	7.4	7.4	0					
10 *H. lanii*	15.4	6.5	6.2	6.7	6.7	6.7	6.2	5.8	4.9	0				
11 *H. lanii*	14.9	6.9	6.7	6.9	7.2	6.9	6.5	6.0	4.9	0.4	0			
12 *H. lanii*	15.1	6.7	6.5	6.7	6.9	6.7	6.2	5.8	5.1	0.2	0.2	0		
13 *H. parabelopolskyi*	18.1	7.2	6.9	6.9	6.9	9.1	8.4	8.6	6.0	5.3	5.8	5.5	0	
14 *H. payevskyi*	16.5	5.8	5.5	5.3	5.3	6.7	5.5	5.8	5.1	5.1	5.1	4.9	4.9	0

[a] The species are numbered as in Fig. 5, in which GenBank accession numbers of their lineages are given. The sequence divergence was calculated with the use of a Jukes–Cantor model of substitutions. The name of new species is given in bold.

Table 3 The sequence divergence (in percentage) between mitochondrial cytochrome *b* lineages of positively identified species of *Plasmodium*

Species[a]	15	16	17	18	19	20	21	22	23	24	25	26	27
15 ***P. globularis***	0												
16 *P. ashfordi*	7.9	0											
17 *P. (Novyella)* sp.	8.6	2.4	0										
18 *P. gallinaceum*	10.6	9.1	9.3	0									
19 *P. circumflexum*	7.4	6.7	6.5	5.1	0								
20 ***P. megaglobularis***	8.6	7.7	7.9	5.8	4.4	0							
21 *P. cathemerium*	7.2	7.2	6.9	4.6	3.3	3.3	0						
22 *P. relictum*	7.9	7.9	8.4	6.2	4.2	3.7	3.3	0					
23 *P. relictum*	8.1	7.7	8.1	6.0	4.4	3.5	3.1	0.2	0				
24 *P. relictum*	7.7	7.2	7.7	5.5	3.9	3.9	3.1	2.4	2.2	0			
25 *P. relictum*	7.9	7.4	7.9	5.8	4.2	4.2	3.3	2.6	2.4	0.6	0		
26 *P. elongatum*	8.8	7.2	7.7	6.7	6.0	6.9	6.0	6.9	6.7	5.8	6.0	0	
27 *P. elongatum*	8.8	7.2	7.7	6.7	6.0	6.9	6.0	6.9	6.7	5.8	6.0	0.0	0

[a] The species are numbered as in Fig. 5, in which GenBank accession numbers of their lineages are given. The sequence divergence was calculated with the use of a Jukes–Cantor model of substitutions. The names of new species are given in bold.

University, Vilnius, Lithuania. Parahapantotypes (accession nos. 41644 NS, USNPC 100948 and G465170) are deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania, in the US National Parasite Collection, Beltsville, USA, and in the Queensland Museum, Queensland, Australia, respectively. Simultaneous infection of *Trypanosoma* sp. was present in the type material.

Etymology The species name is derived from English word 'globule.' This name reflects the presence of one blue, non-refractive globule in erythrocytic meronts of this parasite and the globular-like appearance of cytoplasm in gametocytes, particularly in microgametocytes.

Trophozoites (Fig. 1a,b) are seen in mature erythrocytes; they can be found anywhere in the host cells but are more often seen in sub-polar or lateral position to the nuclei of erythrocytes. The smallest trophozoites are variable in shape, usually irregular or slightly ameboid in outline, but outgrowths extending beyond the main body of the trophozoites are absent; they do not adhere to the nuclei of erythrocytes (Fig. 1a). The 'ring' stage is not characteristic. The parasite nucleus is small, usually central in position (Fig. 1a); vacuoles are not seen. Advanced trophozoites frequently are appressed to the nuclei of infected erythrocytes; they usually locate laterally to the nuclei (Fig. 1b). One prominent blue, non-refractive globule appears in each advanced trophozoite (Fig. 1b); it remains intact in trophozoites and in developing and mature erythrocytic meronts. Fully grown trophozoites usually possess one or two minute brown pigment granules, which are usually located close to the globules; due to small size, it is difficult to distinguish the pigment granules even in advanced trophozoites. The influence of trophozoites on the morphology of infected erythrocytes is not pronounced.

Erythrocytic meronts (Fig. 1c–i; Table 1) are seen only in mature erythrocytes even during heavy infections; the cytoplasm is scanty, stains pale blue and becomes invisible in fully grown meronts; nuclei decrease in size as the parasite matures; vacuoles are not seen. Binuclear developing meronts usually locate laterally to nuclei of the erythrocytes; they frequently adhere to the nuclei (Fig. 1c,d); a blue non-refractive globule was seen in the central (Fig. 1d) and terminal (Fig. 1c) positions. Fully grown meronts are irregular (Fig. 1g), oval elongate (Fig. 1h) or oval (Fig. 1f) in order of frequency of their occurrence in the type material; nuclei are arranged as two rows (Fig. 1f,h) or are scattered (Fig. 1g) in fully grown meronts. Mature meronts with four merozoites represent over 99.9% of all meronts; a few mature meronts with five merozoites were recorded, which is probably an artefact of development. Fan-like meronts were not seen. Pigment granules are brown in colour; between one and two minute-size pigment granules are present in developing meronts; they frequently locate close to the blue globule and are difficult to distinguish. In mature meronts, pigment granules usually are aggregated into a prominent mass (Fig. 1g,h), which is easily distinguishable and usually is located close to the blue globule. Mature meronts usually are not appressed to the nuclei of infected erythrocytes; they can be seen anywhere in the erythrocytes but are more frequently recorded in sub-polar and lateral positions to the host cell nuclei (Fig. 1f–i). Mature merozoites do not exceed 1 μm in diameter, and their cytoplasm is usually invisible (Fig. 1i). The influence of meronts on the morphology of infected erythrocytes usually is not pronounced.

Macrogametocytes (Fig. 1j–o) are seen only in mature erythrocytes even during heavy parasitaemia; the cytoplasm is markedly granular in appearance with large unstained

areas (Fig. 1n), usually lacking vacuoles. Gametocytes are elongated in form and irregular in outline from the earliest stages of their development (Fig. 1j). Growing gametocytes take a position lateral to the nuclei of infected erythrocytes; they frequently are appressed to the nuclei (Fig. 1j–m). Mature gametocytes frequently do not touch the erythrocyte nuclei or envelope or both (Fig. 1m–o); they usually do not fill up the poles of erythrocytes (Fig. 1o). The parasite nucleus is diffuse with its boundaries hardly visible; it is usually central or sub-central in position (Fig. 1m–o). Pigment granules are roundish or oval in form, of small (<0.5 μm) or medium (0.5–1.0 μm) size, usually clumped together into one or several groups, which can be seen anywhere in the gametocytes (Fig. 1k,o). The influence of gametocytes on the morphology of infected erythrocytes usually is not pronounced, but nuclei of some infected erythrocytes may be slightly displaced laterally (Fig. 1m; Table 1).

For microgametocytes (Fig. 1p–t), the general configuration and other features are as for macrogametocytes with the usual haemosporidian sexual dimorphic characters; granular appearance of cytoplasm is better evident than in macrogametocytes. The cytoplasm usually appears as a markedly granulated structure with intermediate unstained areas (Fig. 1r,s); it often looks like the aggregation of numerous roundish, closely packed globules (Fig. 1t), which is a rare character for gametocytes of avian malaria parasites.

Remarks *Plasmodium globularis* belongs to the sub-genus *Novyella*, which unites the avian malaria parasites with small erythrocytic meronts and elongated gametocytes (Corradetti et al. 1963; Garnham 1966; Valkiūnas 2005). Ten species of *Novyella* have been described (Valkiūnas et al. 2007b). Only two of them produce strictly four merozoites in their erythrocytic meronts. These are *P. rouxi* Sergent et al., 1928 and *P. bertii* Gabaldon and Ulloa, 1981. *P. globularis* can be readily distinguished from both these species primarily by (1) the presence of one blue, non-refractive globule in each of its trophozoites and erythrocytic meronts and (2) markedly granular and an even globular-like appearance of the cytoplasm of its gametocytes, particularly microgametocytes (Fig. 1t).

Trophozoites and erythrocytic meronts of *P. bertii* do not possess globules (Gabaldon and Ulloa 1981). Additionally, mature gametocytes of this parasite markedly displace nuclei of infected erythrocytes, frequently to the envelope of the erythrocytes, which is not characteristic of *P. globularis* (Fig. 1).

P. globularis is most similar to *P. rouxi*, a common parasite of passeriform birds (Bishop and Bennett 1992). Developing binuclear erythrocytic meronts of both these species frequently take a bilobular ('bow-tie') form (Figs. 1d and 2a), which is a characteristic feature of development of these parasites. Erythrocytic meronts of both species also possess globules (Sergent et al. 1928; Garnham 1966; Valkiūnas 2005). However, globules in erythrocytic meronts of *P. rouxi* are colourless or of light-turquoise colour (Fig. 2c); they are markedly refractive in appearance (Fig. 2a–c). Additionally, such globules are absent from trophozoites of *P. rouxi*. In *P. globularis*, one blue globule is present in each advanced trophozoite and each erythrocytic meront (Fig. 1b–i); the globule is not refractive in appearance (Fig. 1c–i). Furthermore, these species can be readily distinguished due to the granular appearance of the cytoplasm of gametocytes of *P. globularis*, which is not characteristic of *P. rouxi* (compare Figs. 1o,t and 2d).

Plasmodium (Novyella) megaglobularis n. sp.

Type host The host is the olive sunbird *C. olivacea* (Passeriformes, Nectariniidae).

DNA sequences The DNA sequence is mitochondrial cyt *b* lineage pCYOL1 (468 bp, GenBank accession no. EU770152).

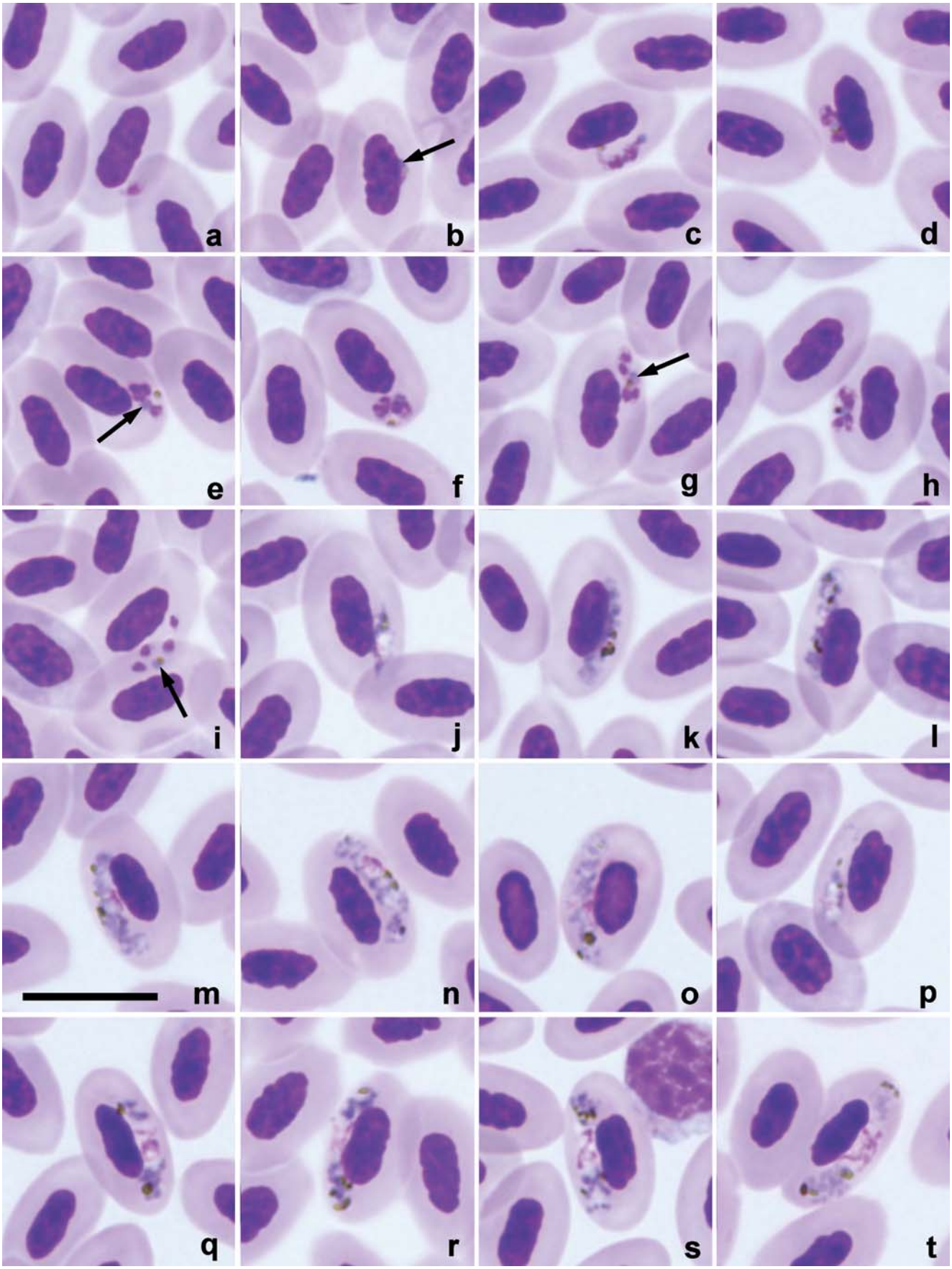
Additional hosts The identical mitochondrial cyt *b* lineage of *Plasmodium* sp. has been recorded in sunbirds in Nigeria (DQ847268, pygmy sunbird *Hedydipna platura*) and Gabon (DQ839054, live-bellied sunbird *Cinnyris chloropygius*, and DQ659570, olive sunbird). It is probable that *P. megaglobularis* parasitise numerous species of the Nectariniidae.

Type locality Its locality is Abrafo, Ghana (05°21.171′ N, 01°23.406′ E, 170 m above sea level).

Prevalence Overall prevalence in the olive sunbird was 94 of 449 (20.9%). In the type locality, the prevalence was 11 of 30 (36.7%). According to microscopic examination and PCR diagnostics, 61.7% of all recorded infections of *P. megaglobularis* were mixed; among these simultaneous infections, 39.4% were with other *Plasmodium* spp., 16% with *Haemoproteus* spp. and 6.4% with both *Plasmodium* spp. and *Haemoproteus* spp. Only samples with single infections were used for the description of *P. megaglobularis.*

Distribution This morphospecies and its lineage pCYOL1 as well as lineages identical to the latter lineage (see "Additional hosts") have been recorded in Ghana, Nigeria, Cameroon and Gabon. They have not been found in European migrants in Africa and after their arrival from

Fig. 1 *Plasmodium* (*Novyella*) *globularis* sp. nov. (lineage pANLA1) from the blood of yellow-whiskered greenbul *Andropadus latirostris*: **a–b** trophozoites; **c–i** erythrocytic meronts; **j–o** macrogametocytes; **p–t** microgametocytes. *Arrows*—blue, non-refractive globule. Giemsa-stained thin blood films. *Scale bar*=10 μm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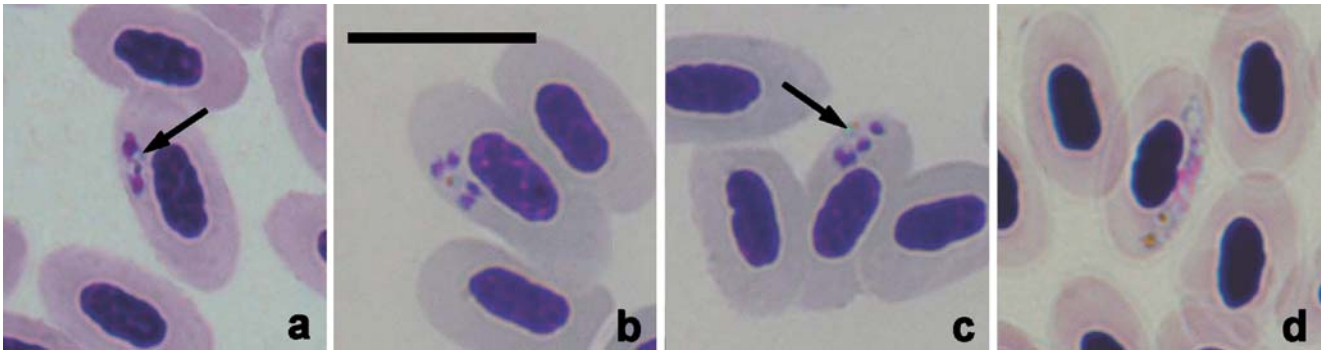

Fig. 2 *Plasmodium* (*Novyella*) *rouxi* from its type host the Spanish sparrow *Passer hispaniolensis* (a passage to canary): **a**–**c** erythrocytic meronts; **d** macrogametocyte. *Arrows*—light-turquoise, markedly refractive globule. Giemsa-stained thin blood films. *Scale bar*=10 μm

African wintering grounds, so it is possible that *P. megaglobularis* is restricted to sunbirds and is transmitted throughout the range of these birds in Africa.

Site of infection Sites of infection are mature erythrocytes; no other data was available.

Type specimens The hapantotype (accession number 42128 NS, intensity of parasitaemia is 0.01%, *C. olivacea*, Abrafo, Ghana, collected by G. Valkiūnas, 14 July 2007) is deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania. Parahapantotypes (accession nos. 42129 NS, USNPC 100947.01 and G465168) are deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania, in the US National Parasite Collection, Beltsville, USA, and in the Queensland Museum, Queensland, Australia, respectively. Simultaneous infection of *Trypanosoma* sp. was present in the type material.

Etymology The species name is derived from the English word 'globule.' The name *megaglobularis* reflects the presence of one large, blue, non-refractive globule in trophozoites and erythrocytic meronts and the markedly globular-like appearance of the cytoplasm both in macro- and microgametocytes. It also reflects morphological similarity of blood stages of this parasite to *P. globularis*. The general appearance of globules in erythrocytic meronts and gametocytes of this parasite is similar to the same structures in *P. globularis*, but the globules are larger in size in the erythrocytic meronts (Table 1) and are more prominent in the gametocytes of *P. megaglobularis*.

Trophozoites (Fig. 3a–b) are seen in mature erythrocytes. The earliest forms can be found anywhere in the host cells but are more often present in the lateral position to the nuclei of the erythrocytes and do not adhere to the nuclei (Fig. 3a); they are variable in shape, but an ameboid outline is not characteristic; the 'ring' stage was not seen; the parasite nucleus is small, usually terminal in position (Fig. 3a); vacuoles are not seen. Advanced trophozoites usually do not adhere to the nuclei of erythrocytes; each trophozoite possesses a large nucleus, a large blue, non-refractive globule, which adheres to the nucleus (Fig. 3b). The globule appears in each advanced trophozoite; it remains intact in trophozoites and in developing and mature erythrocytic meronts. Fully grown trophozoites usually possess one or two prominent brown or black pigment granules, which are usually located close to the globule (Fig. 3b). The influence of trophozoites on the morphology of infected erythrocytes is not pronounced.

Erythrocytic meronts (Fig. 3c–h) are seen only in mature erythrocytes even during heavy infections; the cytoplasm is scanty, stains pale blue and becomes invisible in fully grown meronts; nuclei decrease in size as the parasite matures; vacuoles are not seen. Binuclear developing meronts are markedly variable in shape (Fig. 3c–e); 'bow-tie' forms are present (Fig. 3d). Meronts were seen in polar (Fig. 3c,e) or sub-polar (Fig. 3d, f–h) position in infected erythrocytes; they usually do not adhere (Fig. 3d) or only slightly adhere to the nuclei of erythrocytes (Fig. 3f-h); a large blue, non-refractive globule more frequently was seen in the terminal position (Fig. 3c,e,f,h) but is also present in the central position (Fig. 3d). Fully grown meronts usually are irregular (Fig. 3f,h) in shape; nuclei are randomly scattered (Fig. 3f,h) or arranged as two rows (Fig. 3g). Fan-like meronts were not seen. Mature meronts possess strictly four merozoites (Fig. 3f–h). Pigment granules are brown in colour; between one and three small pigment granules are present in developing meronts; they usually locate close to the blue globule, but there are some exceptions (Fig. 3c). In mature meronts, pigment granules usually are clumped together (Fig. 3f,g) or aggregated into a mass (Fig. 3h); they locate close to the globules. Mature meronts usually are not appressed to the nuclei of infected erythrocytes; they were seen mainly in the sub-polar position (Fig. 3f–h) in infected erythrocytes. Mature merozoites do not exceed 1 μm in diameter, and their cytoplasm is usually invisible (Fig. 3h). The influence of meronts on the morphology of infected erythrocytes usually is not pronounced.

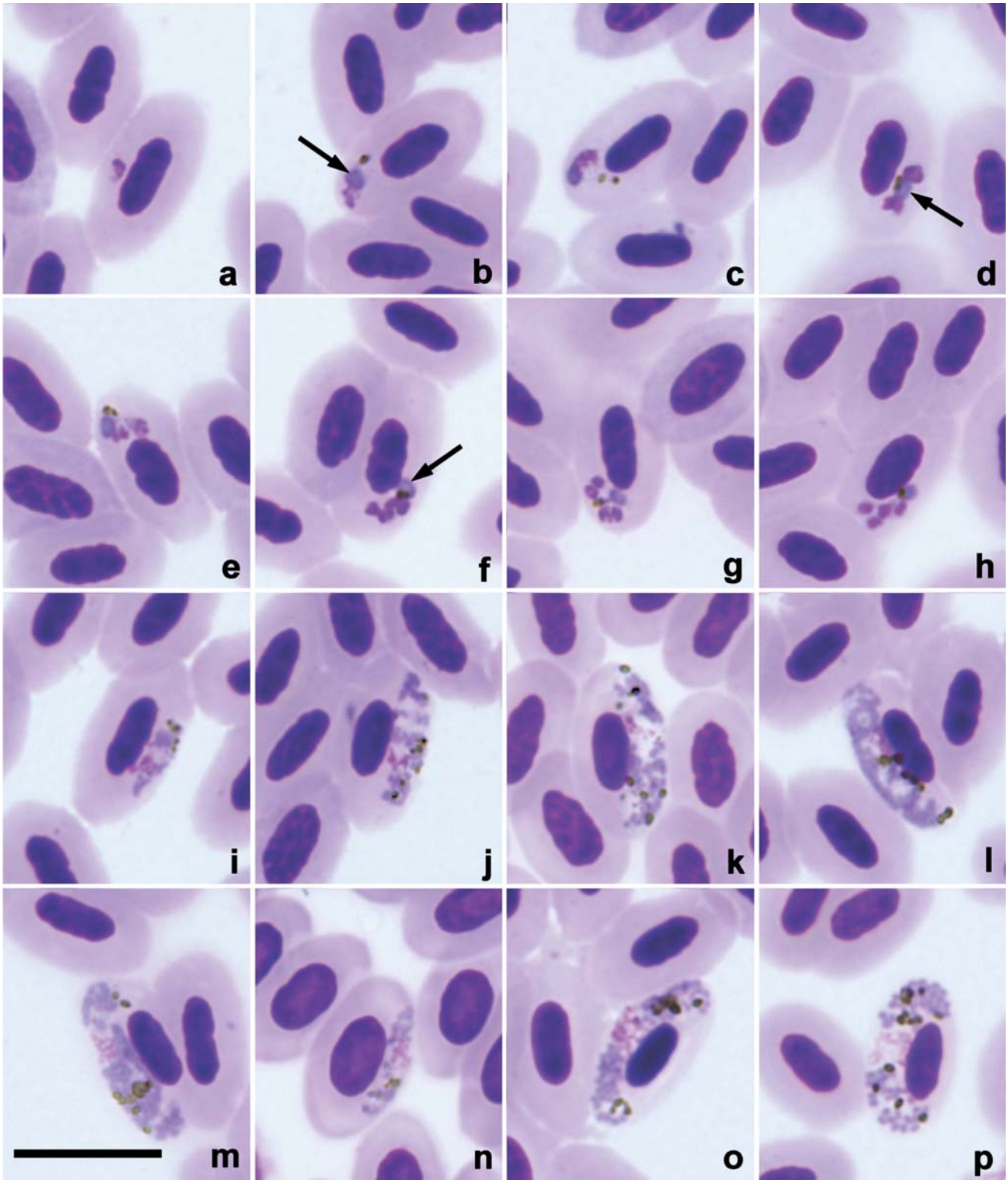

Fig. 3 *Plasmodium* (*Novyella*) *megaglobularis* sp. nov. (lineage pCYOL1) from the blood of olive sunbird *Cyanomitra olivacea*: **a–b** trophozoites; **c–h** erythrocytic meronts; **i–m** macrogametocytes; **n–p** microgametocytes. *Arrows*—large blue, non-refractive globule. Giemsa-stained thin blood films. *Scale bar*=10 μm

Macrogametocytes (Fig. 3i–m) are seen only in mature erythrocytes even during heavy parasitaemia; the cytoplasm is markedly granular in appearance with large unstained areas (Fig. 3j–l), usually lacking vacuoles. The granulation of cytoplasm is well evident; it looks like an irregular package of numerous oval or circular granules with unstained spaces between them; such appearance of the cytoplasm is a characteristic feature of this species (Fig. 3k). Gametocytes are elongated in form and irregular in outline from the earliest stages of their development (Fig. 3i). Growing gametocytes take a position lateral to the nuclei of infected erythrocytes; they are appressed to the nuclei (Fig. 3i). Mature gametocytes are appressed both to the nuclei and envelope of infected erythrocytes; they sometimes fill up the poles of erythrocytes (Fig. 3m). The parasite nucleus is small (Table 1), usually central or sub-central in position (Fig. 3i–m); it is more frequently seen located close to the erythrocyte nucleus, with its boundaries hardly visible (Fig. 3j–l). Pigment granules are roundish or oval, of small (0.5 μm) and medium (0.5–1.0 μm) size; they were seen randomly scattered in the cytoplasm (Fig. 3k) or clumped together into one or several groups which can be found anywhere in the gametocytes (Fig. 3l,m). Mature gametocytes slightly displace the nuclei of erythrocytes (Fig. 3l,m).

For microgametocytes (Fig. 3n–p), the general configuration and other features are as for macrogametocytes with the usual haemosporidian sexual dimorphic characters; the granular appearance of cytoplasm is better evident than in macrogametocytes (Fig. 3p). Other features are as for macrogametocytes.

Remarks As *P. globularis*, *P. megaglobularis* belongs to the sub-genus *Novyella*. By morphology of its blood stages, it is similar to *P. rouxi* and *P. bertii*. *P. megaglobularis* can be readily distinguished from these parasites by the same characters, as *P. globularis* (see "Remarks" to *P. globularis*).

P. megaglobularis (Fig. 3) is most similar to *P. globularis* (Fig. 1). The former species can be readily distinguished, primarily due to (1) large size of globules in its trophozoites and erythrocytic meronts (Table 1; $P<0.001$), (2) markedly globular-like appearance of the cytoplasm both of macro- and microgametocytes (Fig. 3k,p) and (3) approximately half the number of pigment granules in gametocytes (Table 1, $P<0.001$). These characters can be easily distinguished even during light chronic parasitaemias when just a few parasites are present in each blood film. It is important to note that *P. megaglobularis* is a larger parasite than *P. globularis* in all morphometric parameters of its blood stages (Table 1; $P<0.05$ for all measurements of gametocytes and meronts), except for the number of merozoites in their mature erythrocytic meronts. However, because light chronic parasitaemias predominate in sunbirds (more than 80% of all infections), the use of morphometric analyses for these species identification is not always possible.

Haemoproteus (Parahaemoproteus) vacuolatus n. sp.

Type host The host is the yellow-whiskered greenbul *A. latirostris* (Passeriformes, Picnonotidae).

DNA sequences The DNA sequence is mitochondrial cyt *b* lineage hANLA1 (468bp, GenBank accession no. EU770153).

Additional hosts Additional hosts are unknown.

Type locality Its locality is Nkwanta, Ghana (05°16.912′ N, 02°38.495′ E, 85 m above sea level).

Prevalence Overall prevalence in the yellow-whiskered greenbul was 11 of 187 (5.9%). In the type locality, the prevalence was 7 of 45 (17.5%). According to microscopic examination and PCR diagnostics, three of all recorded infections of *H. vacuolatus* were mixed with *P. globularis*. Only samples with single infection were used for description of *H. vacuolatus*.

Distribution This morphospecies and its lineage hANLA1 have been recorded in Ghana and Cameroon so far. It is probable that it is transmitted throughout the range of the yellow-whiskered greenbul in Africa.

Site of infection The sites of infection are mature erythrocytes; no other data was available.

Type specimens The hapantotype (accession number 42415 NS, intensity of parasitemia is 0.01%, *A. latirostris*, Nkwanta, Ghana, collected by G. Valkiūnas, 19 July 2007) is deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania. Parahapantotypes (accession nos. 42416 NS, USNPC 100946.01 and G465166) are deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania, in the US National Parasite Collection, Beltsville, USA, and in the Queensland Museum, Queensland, Australia, respectively.

Etymology The species name is derived from English word 'vacuole.' This name reflects the presence of one prominent vacuole in the cytoplasm of each advanced macrogametocyte.

For young gametocytes (Fig. 4a), the earliest forms were not seen in type material. As the parasite develops, gametocytes adhere to the nucleus of erythrocytes and extend longitudinally along the nuclei but do not touch the envelope of erythrocytes along their entire margin. Growing gametocytes, which exceed the length of nuclei of

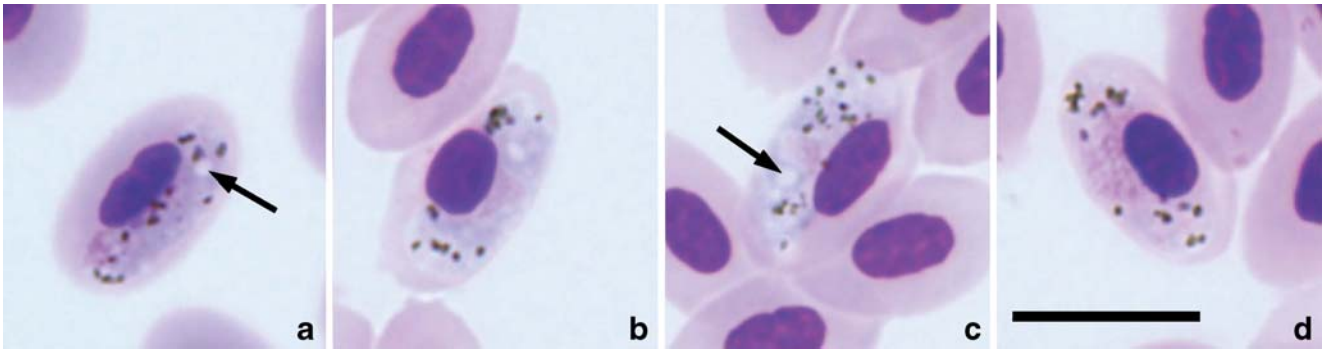

Fig. 4 *Haemoproteus* (*Parahaemoproteus*) *vacuolatus* sp. nov. (lineage hANLA1) from the blood of yellow-whiskered greenbul *Andropadus latirostris*: **a–c** macrogametocytes; **d** microgametocyte. *Arrows*—large vacuole. Giemsa-stained thin blood films. *Scale bar*=10 μm

erythrocytes, usually do not touch the envelope of erythrocytes along their entire margin (Fig. 4a). This is a characteristic feature of development of this species. The outline is even or slightly irregular.

Macrogametocytes (Fig. 4b,c) develop in mature erythrocytes; the cytoplasm is blue, homogenous in appearance, lacking valutin granules. A clear discrete vacuole is present in each gametocyte (Fig. 4a–c). Gametocytes grow along the nuclei of infected erythrocytes; they slightly enclose the nuclei with their ends but do not encircle them completely. Fully grown gametocytes are closely appressed to the nuclei and envelope of erythrocytes. The parasite nucleus is variable in form, frequently band-like in shape. The nucleus has no permanent position in gametocytes; it usually is seen in a position lateral to the nucleus of erythrocyte; it adheres to the nucleus (Fig. 4b). In growing gametocytes, the parasite nucleus was also recorded in the terminal position (Fig. 4a). A nucleolus was not seen. Pigment granules are of small (<0.5 μm) and medium (0.5–1 μm) size, roundish and oval, usually randomly scattered throughout the cytoplasm (Fig. 4a,c) but sometimes are clumped at the ends of gametocytes (Fig. 4b). Nuclei of infected erythrocytes are slightly displaced laterally (Fig. 4c; Table 1).

For microgametocytes (Fig. 4d) , the general configuration is as for macrogametocytes with the usual haemosporidian sexually dimorphic characters. Typical for macrogametocytes, vacuoles are absent from microgametocytes.

Remarks Vacuoles are frequently present in gametocytes of avian haemoproteids; the number of vacuoles usually is variable in different gametocytes of the same species (Valkiūnas 2005). *H. vacuolatus* can be readily distinguished from all species of haemoproteids of passeriform birds due to one clear vacuole, which is present in each advanced macrogametocyte (Fig. 4a–c). A similar distinctive vacuole also occurs in macrogametocytes of *Haemoproteus gavrilovi* Valkiūnas and Iezhova, 1990, *H. ortalidum* Gabaldon and Ulloa, 1978 and *H. trogonis* Bennett and Peirce, 1990; these haemoproteids parasitise birds belonging to the Coraciiformes, Galliformes and Trogoniformes, respectively. Mature gametocytes of *H. gavrilovi* enucleate host cells (Valkiūnas 2005); mature gametocytes of *H. ortalidum* are cylindrical and even discoid in shape (Gabaldon and Ulloa 1978); vacuoles are clearly nucleophilic in macrogametocytes of *H. trogonis* (Bennett and Peirce 1990). None of these features are characteristic of *H. vacuolatus*.

During the identification of *H. vacuolatus*, attention also should be paid to the variable position of the nucleus in the macrogametocyte, which is an uncommon feature for haemoproteids of passeriform birds.

Phylogenetic relationships of parasites

The lineage pANLA1 of *P. globularis* and the lineage pCYOL1 of *P. megaglobularis* belong to clearly different clades (Fig. 5), with sequence divergence between these two lineages of more than 8% (Table 3). Because both parasites belong to the sub-genus *Novyella*, this finding contradicts the data on the morphology of these similar malaria parasites (Figs. 1 and 3) and the traditional taxonomy of *Plasmodium* spp. on the level of sub-genera.

The lineage pANLA1 clusters well with the lineage pGRW2 of *Plasmodium ashfordi* Valkiūnas et al. 2007 (Fig. 5), with a genetic distance more than 7% between these lineages (Table 3). The clade C represents three morphospecies of malaria parasites, which belong to the sub-genus *Novyella*.

The lineage pCYOL1 of *P. megaglobularis* belongs to a diverse group of lineages (clade D in Fig. 5) containing ten other lineages of positively identified morphospecies of *Plasmodium*, with a genetic distance between them of 3.3–5.8% (Table 3). Parasites of this well-supported clade belong to three sub-genera of avian malaria parasites, i.e. *Haemamoeba* (*Plasmodium gallinaceum*, *P. cathemerium*, *P. relictum*), *Giovannolaia* (*P. circumflexum*) and *Novyella* (*P. megaglobularis*; Fig. 5).

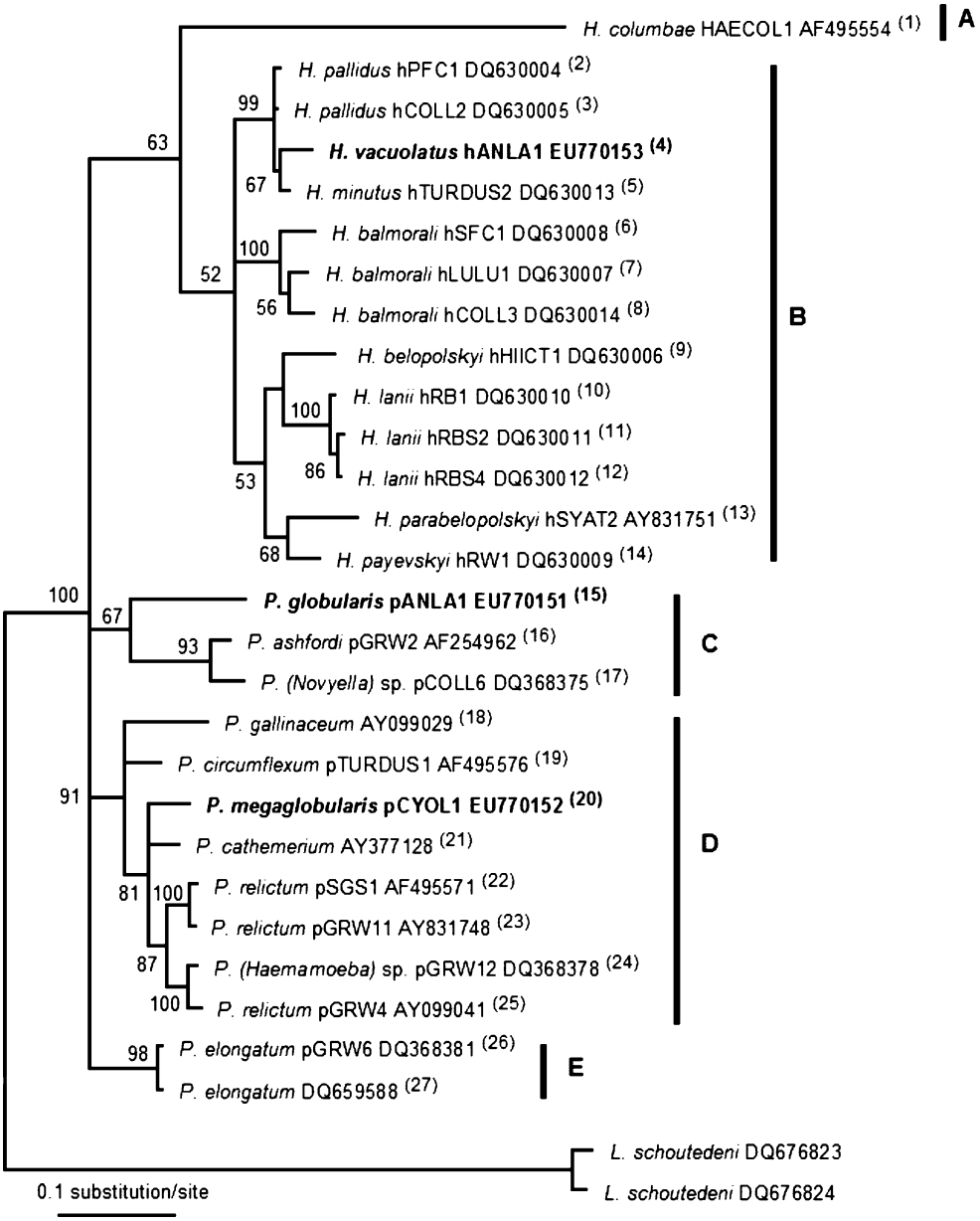

Fig. 5 Bayesian phylogeny of 14 mitochondrial cytochrome *b* lineages of *Haemoproteus* spp., 13 lineages of *Plasmodium* spp. and two lineages of *Leucocytozoon schoutedeni* used as outgroups. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the parasite species names. The lineages are numbered (ciphers in parentheses) as in Tables 2 and 3. *Vertical bars* indicate species of the sub-genera *Haemoproteus* (*A*), *Parahaemoproteus* (*B*), *Novyella* (*C*), and *Huffia* (*E*). The bar *D* indicates a group of closely related lineages of *Plasmodium* spp. belonging to the sub-genera *Giovannolaia* (*P. circumflexum*), *Novyella* (*P. megaglobularis*) and *Haemamoeba* (all other species). The names of new species are given in bold

The lineage hANLA1 of *H. vacuolatus* belongs to a diverse group of lineages (clade B in Fig. 5) containing 13 other lineages of positively identified morphospecies of haemoproteids of the sub-genus *Parahaemoproteus*. It clusters well with lineages of *Haemoproteus pallidus* and *H. minutus*. Genetic divergence between lineages of these three morphologically well-differentiated parasites is less than 2% (Table 2).

The majority of positively identified species of different sub-genera of avian haemosporidian parasites belong to clearly different clades in the phylogenetic tree (Fig. 5, clades A–E), in accordance with their morphological and biological characteristics. Exceptions are *P.* (*Giovannolaia*) *circumflexum* and *P.* (*Novyella*) *megaglobularis*, which cluster with species of the sub-genus *Haemamoeba*.

Discussion

Identifying avian haemosporidian parasites to the morphospecies level is a complex task mainly because parasitaemias are light in wildlife. Acute infections of these haematozoa are frequently pathogenic, so they typically are under-sampled using mist netting (Valkiūnas 2005). Molecular diagnostics of haemosporidians should improve species identification (Palinauskas et al. 2007). However, one should always be aware that the currently used PCR protocols are indirect detection methods. The amplification of DNA fragments by PCR includes not only target DNA but often also DNA from simultaneous infections, so the validation of molecular techniques is important (Pérez-Tris and Bensch 2005). Simultaneous infections with haemato-

zoa belonging to the same and different genera are frequent in wildlife and are commonly found in some bird species, but nested PCR techniques may not always detect these infections (Valkiūnas et al. 2006). Cautious consideration is necessary when interpreting sequence data obtained from naturally infected birds. During these infections, it is difficult to completely rule out the possibility that low-level mixed infections may be present in a sample but may not be detected by microscopy. In this case, a sequence obtained and assumed to be that of the parasite seen under the microscope might in fact be derived from a low-level infection of another species and thus linked with the wrong morphospecies. This must be taken into consideration in molecular systematic and phylogenetic studies and probably accounts for the majority of misidentified morphospecies, whose sequences are deposited in GenBank (Valkiūnas et al. 2008a). In our samples from the olive sunbird, we often discover simultaneous infections. To avoid this problem, we used multiple samples, which were identified as single infections both by PCR-based methods and microscopic examination of entire blood films from the type series.

P. globularis and *P. megaglobularis* are clearly different from other malaria parasites of the sub-genus *Novyella* due to large blue, non-refractive globules, which are present in advanced trophozoites and erythrocytic meronts of these parasites (Garnham 1966; Valkiūnas 2005). The biological function and cellular composition of the globules remain unknown. Because such globules have been recorded only in *Novyella* species, which are transmitted in Africa, it is possible that the development of the globules is a specific adaptation of African *Plasmodium* species. This warrants further investigation.

In spite of the high prevalence of *P. globularis* and *P. megaglobularis* in their type vertebrate hosts, these parasites seem to be highly specific and restricted to birds of the Picnonotidae and Nectariniidae, respectively. High specificity has been reported for several species belonging to the sub-genus *Novyell*a (Iezhova et al. 2005), but mechanisms conferring this specificity remain unknown. There is no published information regarding the vectors of *Novyella* species in sub-Saharan Africa. Further studies are needed to understand the high host specificity of *Novyella* species, and *P. megaglobularis* would be a convenient model organism for such studies since it has high prevalence in the olive sunbird and seems to be widespread in this common African bird.

It is worth noting that *P. globularis* and *P. megaglobularis* have not been recorded in Palearctic birds wintering in Africa. It is probable that the high specificity of these parasites precludes infection of Palearctic birds overwintering in Africa; this may explain the apparent lack of these infections in the Palearctic.

We used only positively identified morphospecies of avian haemosporidians in phylogenetic analyses (Wiersch et al. 2005; Sehgal et al. 2006b; Hellgren et al. 2007; Palinauskas et al. 2007; Valkiūnas et al. 2007a, b; Valkiūnas et al. 2008d). Sequences of three positively identified species of *Plasmodium* from the sub-genus *Novyella* are available in GenBank, i.e. *P. ashfordi*, *P. globularis* and *P. megaglobularis* (Fig. 5). Mitochondrial cyt *b* lineages of *P. ashfordi* and *P. globularis* cluster together but not with *P. megaglobularis*. These data support the described paraphyly of the sub-genus *Novyella* (Martinsen et al. 2007); however, the morphospecies of the *Novyella* were not identified in that study, and the probability of the presence of simultaneous infections was not ruled out completely. The following explanations may explain our finding. First, the current sub-generic classification of avian malaria parasites (Corradetti et al. 1963; Garnham 1966; Valkiūnas 2005) might not reflect the phylogenetic relationships among avian *Plasmodium* species and should be re-worked. Second, phylogenies, which are based on mitochondrial genes, do not reflect or reflect only in part the phylogenetic relationships among avian haemosporidian parasites on the level of their species and sub-genera. It is worth noting that the genus *Plasmodium* was viewed as paraphyletic relative to the genus *Haemoproteus* in a mitochondrial cyt *b* gene phylogeny (Perkins and Schall 2002) but was monophyletic in a recent four-gene analysis (Martinsen et al. 2008). To test both hypotheses, additional studies are required using multi-gene analyses and more lineages from positively identified morphospecies of haemosporidian parasites. It should also be noted that taxonomic boundaries among the sub-genera *Haemamoeba* and *Giovannolaia* and *Novyella* and *Giovannolaia* are not strict and the positions of some species of avian malaria parasites in these sub-genera are not clear and even provisional. For instance, erythrocytic meronts of *P.* (*Giovannolaia*) *garnhami* Guindy, Hoogstraal and Mohammed, 1965 produce six to eight merozoites, so this species is more closely related to species of *Novyella* based on this feature (Garnham 1966). Investigations on the phylogeny of haemosporidians would be more convincing if the type species and morphologically similar species from each sub-genus would be used to confirm the validity of the current sub-generic classification of avian malaria parasites. This classification reflects morphological diversity of avian *Plasmodium* spp., is helpful for species identification and should be developed carefully.

H. vacuolatus was attributed tentatively to the sub-genus *Parahaemoproteus* because its lineage hANLA1 clusters well with other avian species of the sub-genus *Parahaemoproteus* but not to the lineages of *Haemoproteus columbae* Kruse, 1890 (Fig. 5), which belongs to the sub-genus *Haemoproteus*. Haemoproteids of the sub-genera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of vectors and undergo markedly different sporogony (Garnham 1966; Valkiūnas et al. 2002). Bennett et al. (1965)

established the genus *Parahaemoproteus* for avian haemoproteids, which are characterised by small oocysts (<20 μm in diameter) that possess one germinative centre, a relatively small number of sporozoites in mature oocysts (<100), relatively long sporozoites that are usually pointed at both ends (mean length is usually greater than 10 μm), transmitted by biting midges belonging to the Ceratopogonidae and some other features (Valkiūnas 2005). Haemoproteids, which are transmitted by hippoboscid flies belonging to the Hippoboscidae, were placed to the sub-genus *Haemoproteus*; they are characterised by large oocysts (>20 μm in diameter) that possess numerous germinative centres, relatively many sporozoites in mature oocysts (>500), relatively short sporozoites (mean less than 10 μm) that are usually blunt at one end and pointed in the other and some other features (Garnham 1966; Valkiūnas 2005). It worth noting that species of the sub-genus *Haemoproteus* have been recorded only in columbiform birds, but there probably might be exceptions (see Valkiūnas 2005, p. 861).

Levine and Campbell (1971) suggested a sub-generic classification of avian haemoproteids. Two sub-genera were established in the genus *Haemoproteus*; *Parahaemoproteus* was considered as a sub-genus of the genus *Haemoproteus*. Haemoproteids, which are transmitted by hippoboscid flies, were placed to the sub-genus *Haemoproteus*. In spite of clear differences between species of *Haemoproteus* and *Parahaemoproteus*, the establishment of the sub-generic classification of avian haemoproteids (Levine and Campbell 1971) was widely accepted in taxonomic literature, including publications of the authors of the genus *Parahaemoproteus* (Bishop and Bennett 1992). That simplified the generic identification of species whose life cycles were studied incompletely, especially in the vectors, so it provided an opportunity to use binomial nomenclature. The sub-generic classification of haemoproteids also was in accord with widely used sub-generic classification of *Plasmodium* spp. (Garnham 1966). All that was convenient for faunistic and applied research. This convenient device contributed to the development of taxonomy of haemoproteids at the species level but did not solve the problem regarding the phylogenetic relationships between *Parahaemoproteus* and *Haemoproteus*; this problem was only transferred to the level of sub-genera, which has significantly less influence on nomenclature.

Resent molecular findings show that species of *Parahaemoproteus* and *Haemoproteus* might be distinguished due to their position on phylogenetic trees, which are constructed using mitochondrial gene sequences (Hellgren et al. 2007; Valkiūnas et al. 2007a; Martinsen et al. 2008). If so, the use of mitochondrial sequences would be a convenient opportunity to distinguish species of *Parahaemoproteus* and *Haemoproteus*. Only one species of the sub-genus *Haemoproteus*, i.e. *H. columbae*, usually has been used in resent molecular phylogenies of haemosporidians (Fig. 5). The position of other species of the sub-genus *Haemoproteus* in the phylogenetic tree remained unknown. Surprisingly, *Haemoproteus* (*Haemoproteus*) *turtur* Covaleda Ortega and Gállego Berenguer, 1950, a common parasite of doves, appeared in the *Parahaemoproteus* clade in a three-genome phylogeny of avian haemosporidians (Martinsen et al. 2008). Because *H. turtur* completes development in hippoboscid flies (Rashdan 1998), it might be that molecular identification of hippoboscid-transmitted haemoproteids using currently applied molecular markers might be unreliable. Sequences of other avian haemoproteids, which are transmitted by hippoboscids, particularly *Haemoproteus* (*Haemoproteus*) *sacharovi* Novy and MacNeal, 1904, *Haemoproteus* (*Haemoproteus*) *palumbis* Baker, 1966 and additional samples from *H. columbae* and *H. turtur* are needed to clarify this issue. Because lineages of *H. turtur* clustered with ceratopogonid transmitted species of *Parahaemoproteus* (Martinsen et al. 2008), a possibility for this parasite to develop in biting midges should be also tested.

It is worth noting that genetic distance between morphologically well-differentiated *H. minutus*, *H. pallidus* and *H. vacuolatus* is small (Table 2). The genetic distance between mitochondrial cyt *b* lineage hCOLL2 of *H. pallidus* and hTURDUS2 of *H. minutus* is only two substitutions or 0.7% (Hellgren et al. 2007). These findings show that even small genetic distances between mitochondrial cyt *b* gene lineages sometimes reflect an inter-species divergence of haemosporidians. In other words, even nearly identical lineages of haemosporidians cannot be attributed to the same morphospecies without morphological investigations. In the same regard, there are several examples of cryptic speciation, where morphological species are evolutionarily quite divergent (Bensch et al. 2004; Sehgal et al. 2006a, b; Palinauskas et al. 2007). Mechanisms contributing to the evolution of such closely related species remain unknown. It is probable that controlled experiments for direct hybridisation of hemosporidians can be used to reconcile molecular and morphological data and to define biological species for this group of parasites (Valkiūnas et al. 2008b).

In conclusion, the present efforts in several laboratories to obtain sequence data from several genes of hemosporidian species is expected to add a substantial number of new genetic lineages. These data will make the phylogenetic trees of these blood parasites more detailed and precise. Before we have more information regarding the phylogenetic relationships of positively identified species of haemosporidians belonging to all sub-genera of the Plasmodiidae and Haemoproteidae, revision of the current taxonomy of the Haemosporida, which is based mainly on morphological and life history characters, would be premature. Further work to increase the number of precise

linkages between haemosporidian DNA lineages with their morphospecies is therefore an urgent task.

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