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HAEMOPROTEUS CYANOMITRAE SP. NOV. (HAEMOSPORIDA: HAEMOPROTEIDAE) FROM A WIDESPREAD AFRICAN SONGBIRD, THE OLIVE SUNBIRD, *CYANOMITRA OLIVACEA*

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ABSTRACT: *Haemoproteus* (*Parahaemoproteus*) *cyanomitrae* n. sp. (Haemosporida: Haemoproteidae) was found in the olive sunbird, *Cyanomitra olivacea* (Nectariniidae), in rain forests of tropical Africa. It is described based on the morphology of its blood stages and a segment of the mitochondrial cytochrome *b* gene (GenBank accession FJ404696), which can be used for molecular identification and diagnosis of this species. Fully grown gametocytes of new parasite are similar to many other species of haemoproteids of passeriform birds, so are not easily distinguishable from many of them at this stage of their development. *Haemoproteus cyanomitrae* can be readily distinguished from the majority of described species of avian haemoproteids, primarily due to its growing dumbbell-shaped gametocytes and advanced halteridial gametocytes, both of which do not touch the envelope of infected erythrocytes along their entire margin. Pigment granules are most frequently clamped in 1, or several, groups in gametocytes, with larger group of pigment granules usually located close to one end of the gametocyte; asymmetric position of pigment granules in gametocytes a characteristic feature of this parasite. Illustrations of blood stages of the new species are given, and phylogenetic analysis identifies DNA lineages closely related to this parasite, which is widespread in African rain forests, but has been recorded only in the olive sunbird so far. It is probable that *H. cyanomitrae* is transmitted throughout the range of the olive sunbird in Africa.

Species of *Haemoproteus* (Haemosporida: Haemoproteidae) are cosmopolitan, dipteran-borne intracellular blood parasites of birds and other vertebrates (Garnham, 1966). They are widespread in birds, with over 140 species described so far (Valkiūnas, 2005; Valkiūnas, Iezhova, Loiseau et al., 2008). Haemoproteids are often considered as being relatively benign to birds (Bennett et al., 1993). However, some species have been reported to cause severe diseases in avian hosts (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002), sometimes even lethal (Ferrell et al., 2007), and they can affect their fitness (Nordling et al., 1998; Marzal et al., 2005; Valkiūnas, 2005). Recent molecular studies have revealed marked diversity and complexity in terms of speciation and host–parasite evolution of haemoproteids and other related haemosporidian parasites (Bensch et al., 2009). However, it is still unclear how many of these lineages are biological species and how many of them represent different levels of intraspecific variation of haemosporidians. GenBank currently contains few named species of avian *Haemoproteus* and other haemosporidian parasites, with the majority of lineages being classified only to the generic level. In addition, several of these parasites are clearly misidentified (Valkiunas, Atkinson et al., 2008). This is unfortunate because linkage between DNA sequences and identifications based on traditional morphological species can provide important knowledge about basic life history strategies for parasitologists and evolutionary biologists studying phylogenetic relationships of these organisms.

During an ongoing study on the effects of deforestation on the prevalence of blood pathogens in African rain forest birds, large numbers of blood samples were collected from a widespread African songbird, the olive sunbird, *Cyanomitra olivacea* (Nectariniidae), in rain forests in Ghana and Cameroon. One previously undescribed species of *Haemoproteus* was found during this study. This parasite is described here by using data on the morphology of its blood stages and the sequences of the mitochondrial cytochrome *b* (cyt *b*) gene.

MATERIAL AND METHODS

Collection of blood samples

Blood samples were collected in Ghana and Cameroon at 25 sites in both rainy and dry seasons during 2005–2007. Study sites were Agumatsa, Abrafo, and Nkwanta in Ghana. The main study sites in Cameroon were described by Bonneaud et al. (2009). In all, samples from 449 adult olive sunbirds were used in this study. The birds were caught with mist nets and banded to avoid resampling. Blood was taken by puncturing the brachial vein; all birds were then released with none of the individuals being recaptured.

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) and then held at ambient temperature in the field and later at −20 C in the laboratory.

Two or 3 blood films were prepared from each bird. Blood films were air-dried within 5–10 sec after their preparation; they 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 preparation, fixation, staining, and microscopic examination of blood films are described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). 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 determine possible presence of simultaneous infections with other haemosporidian parasites in the type material of new species, the entire blood films from hapantotype and parahapantotype series were examined microscopically at low magnification.

Morphological analysis

A BX61 light microscope (Olympus, Tokyo, Japan) equipped with a DP70 digital camera (Olympus) and imaging software

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analySIS FIVE (Olympus Soft Imaging Solution GmBH, Münster, Germany) was used to examine slides, prepare illustrations, and take measurements. The morphometric features studied (Table I) are those defined by Valkiūnas (2005). Morphology of new species was compared with voucher specimens of *Haemoproteus* (*Parahaemoproteus*) *sequeirae* from the blood of the plain-throated sunbird, *Anthrepetes malacensis* (accessions 1778, 5808A, 5808B, and 5808C), in the Collection of the International Reference Centre for Avian Haematozoa at the Queensland Museum, Queensland, Australia. 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, polymerase chain reaction (PCR) amplification, and sequencing

DNA was extracted from whole blood following a DNeasy kit protocol (QIAGEN, Valencia, California). Success of each DNA extraction was verified with primers that amplify the gene encoding the brain-derived neurotrophic factor (Sehgal and Lovette, 2003). For *Haemoproteus* spp. detection, we used a PCR method that amplifies a fragment of the cyt *b* region of the mitochondrial (mt)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 sec, 53 C annealing for 50 sec, and 72 C extension for 60 sec, 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 *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 (United States Biochemical Corporation, Cleveland, Ohio). All lineages were identified by sequencing the PCR products (BigDye® version 1.1 sequencing kit, Applied Biosystems, Foster City, California) 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 *Haemoproteus* deposited in GenBank and in the database MalAvi (Bensch et al., 2009). 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, Atkinson et al., 2008).

Phylogenetic analysis

We used 16 mitochondrial cyt *b* sequences of avian *Haemoproteus* spp. from our survey and GenBank. The GenBank accessions of these sequences are given in Figure 25. The sequences were aligned using Sequencer 4.8 (GeneCodes, Ann Arbor, Michigan). All individual sequences were grouped into a

TABLE I. Morphometry of host cells and mature gametocytes of *Haemoproteus cyanomitrae* sp. nov. from the olive sunbird, *Cyanomitra olivacea*.

Feature	Measurements*
Uninfected erythrocyte	
Length	9.9–11.7 (10.9 ± 0.5)
Width	5.7–7.0 (6.4 ± 0.3)
Area	48.4–61.1 (55.2 ± 3.9)
Uninfected erythrocyte nucleus	
Length	5.3–6.5 (5.7 ± 0.3)
Width	1.9–2.5 (2.3 ± 0.1)
Area	10.0–11.9 (11.1 ± 0.6)
Macrogametocyte	
Infected erythrocyte	
Length	11.3–13.1 (12.4 ± 0.5)
Width	5.2–7.2 (6.1 ± 0.5)
Area	55.3–72.9 (62.0 ± 4.4)
Infected erythrocyte nucleus	
Length	4.8–6.0 (5.6 ± 0.3)
Width	1.6–2.6 (2.0 ± 0.3)
Area	7.8–12.7 (10.0 ± 1.3)
Gametocyte	
Length	13.3–16.7 (14.3 ± 0.8)
Width	1.5–2.7 (2.2 ± 0.3)
Area	29.6–44.2 (35.2 ± 3.6)
Gametocyte nucleus	
Length	1.5–5.2 (2.4 ± 0.8)
Width	1.2–2.5 (1.5 ± 0.3)
Area	1.5–5.0 (2.9 ± 1.0)
Pigment granules	8.0–13.0 (10.5 ± 1.7)
NDR†	0.6–1.0 (0.8 ± 0.1)
Microgametocyte	
Infected erythrocyte	
Length	10.7–13.1(12.0 ± 0.6)
Width	5.3–7.3 (6.4 ± 0.5)
Area	53.1–74.6 (63.3 ± 5.7)
Infected erythrocyte nucleus	
Length	5.2–6.4 (5.6 ± 0.4)
Width	1.7–2.6 (2.1 ± 0.3)
Area	8.3–12.4 (9.9 ± 1.2)
Gametocyte	
Length	13.8–17.8 (15.5 ± 1.2)
Width	1.5–2.9 (2.3 ± 0.4)
Area	28.5–44.7 (35.5 ± 5.1)
Gametocyte nucleus	
Length	4.8–8.5 (6.3 ± 1.0)
Width	1.5–2.9 (2.1 ± 0.4)
Area	6.6–15.6 (11.0 ± 2.7)
Pigment granules	8.0–14.0 (10.7 ± 1.9)
NDR	0.6–0.9 (0.8 ± 0.1)

* All measurements (n = 21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

† NDR = nucleus displacement ration according to Bennett and Campbell (1972).

TABLE II. Sequence divergence (in percentage) between mitochondrial cytochrome *b* lineages of positively identified species of *Haemoproteus*.

Species*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *H. vacuolatus*	0																
2. *H. pallidus*	1.9	0															
3. *H. pallidus*	1.7	0.2	0														
4. *H. minutus*	1.7	0.6	0.4	0													
5. *H. balmorali*	5.3	4.8	4.6	5.1	0												
6. *H. balmorali*	5.5	5.5	5.3	5.8	3.0	0											
7. *H. balmorali*	5.3	5.3	5.1	5.5	2.4	3.0	0										
8. ***H. cyanomitrae***	4.4	3.9	3.7	4.2	3.3	5.1	4.4	0									
9. ***H. cyanomitrae***	4.6	4.2	3.9	4.4	3.5	5.3	4.6	0.2	0								
10. ***H. cyanomitrae***	6.0	5.3	5.1	5.5	5.1	5.8	5.5	2.8	3.0	0							
11. *H. lanii*	6.7	6.5	6.2	6.7	6.2	6.7	5.8	5.8	6.0	6.2	0						
12. *H. lanii*	6.9	6.9	6.7	7.2	6.5	6.9	6.0	5.3	5.5	6.2	0.4	0					
13. *H. lanii*	6.7	6.7	6.5	6.9	6.2	6.7	5.8	5.5	5.8	6.5	0.2	0.2	0				
14. *H. parabelopolskyi*	6.9	7.2	6.9	6.9	8.3	9.1	8.6	7.2	7.4	7.4	5.3	5.8	5.5	0			
15. *H. payevskyi*	5.3	5.8	5.5	6.0	5.5	6.7	5.8	5.1	5.3	6.5	5.1	5.1	4.8	4.8	0		
16. *H. belopolskyi*	5.8	5.5	5.3	5.8	7.4	7.2	7.4	6.2	6.5	6.2	4.8	4.8	5.1	6.0	5.1	0	
17. *H. columbae*	15.9	15.4	15.1	15.6	16.7	16.4	15.9	15.6	15.4	17.2	15.4	14.8	15.1	18.0	16.4	14.3	0

* The species are numbered as in Figure 25, in which GenBank accessions 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.

consensus that was 468 bp. A lineage of *Haemoproteus columbae* was used as an outgroup (see Fig. 25).

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 1 cold of two hot Monte Carlo Markov chains, which were sampled every 200 generations over 20×10^6 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 (Table II) 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).

DESCRIPTION

Haemoproteus (*Parahaemoproteus*) *cyanomitrae* n. sp.

(Figs. 1–20; Table I)

Young gametocytes (Figs. 1–4): Earliest forms were seen anywhere in infected erythrocytes but more frequently recorded lateral to erythrocyte nuclei. Gametocytes, which reach size of nuclei of erythrocytes in length, frequently do not touch both nuclei and envelope of erythrocytes (Fig. 2). Advanced gametocytes extend longitudinally along nuclei of erythrocytes and adhere to nuclei (Figs. 3, 4). Growing gametocytes, which exceed length of erythrocyte nuclei, usually do not touch envelope of erythrocytes along entire margin (Figs. 1, 4), a characteristic feature in development of this species. Pigment granules are small (<0.5 μm) and can be grouped (Figs. 1–4). Outline of growing gametocytes is wavy (Fig. 2), irregular (Figs. 3, 4), or ameboid (Fig. 1).

Macrogametocytes (Figs. 5–l2): Develop in mature erythrocytes. Cytoplasm is pale blue, heterogeneous in appearance, usually markedly granulated in mature gametocytes (see Figs. 8, 12), lacking volutin granules. Gametocytes grow along nuclei of infected erythrocytes, closely touching erythrocyte nuclei from early stages of development (Figs. 4, 5), slightly displace nuclei laterally, enclosed with ends, but do not encircle nuclei completely. Central part of gametocyte pellicle frequently constricted (Figs. 5, 6), presenting dumbbell-like appearance to parasite. Dumbbell-shaped gametocytes predominate among growing macrogametocytes (Figs. 5–7); closely associated with nuclei of erythrocytes. Dumbbell–shaped gametocytes do not touch envelope of erythrocytes along their entire margin (Fig. 5) also present. As parasite matures, dumbbell-shaped gametocytes touch envelope of erythrocytes with 1 (Fig. 6) or both ends (Fig. 7); finally, dumbbell-shaped gametocytes disappear. Advanced gametocytes do not touch envelope of erythrocytes along their entire margin; in host cells with such gametocytes, a clear, more or less, evident unfilled space ('cleft') present between parasites and erythrocyte envelope, a characteristic developmental feature for this species (see Fig. 8). Mature gametocytes often enclose nuclei of erythrocytes with their ends, and do not feel up the poles of erythrocytes (Fig. 10). Fully grown gametocytes closely associated with nuclei and envelope of erythrocytes, filling erythrocytes up to their poles (Fig. 12). Parasite nucleus variable in form, frequently band-like in shape (Figs. 8, 9), usually subterminal in position (Figs. 7–12). Nucleolus not seen. Pigment granules medium sized (0.5–1 μm), roundish, oval, and sometimes slightly elongated in form; may be randomly scattered throughout cytoplasm but most frequently clumped in 1, or several, groups, with larger group of pigment granules usually located close to one end of the gametocyte (Figs. 10, 11); asymmetric position of pigment granules in gametocytes is characteristic. Outline of macrogametocytes varies from even (Figs. 10, 12) to wavy (Figs. 7, 8) and ameboid (Figs. 5, 9) but more frequently the latter. Nuclei of infected erythrocytes slightly displaced laterally (Figs. 5–12, Table I).

Microgametocytes (Figs. 13–20): General configuration as for macrogametocytes with usual haemosporidian sexually dimorphic characters. Pigment granules in mature gametocytes may be aggregated in solid mass (Fig. 19).

Taxonomic summary

Type host: Cyanomitra olivacea L. (Passeriformes, Nectariniidae).

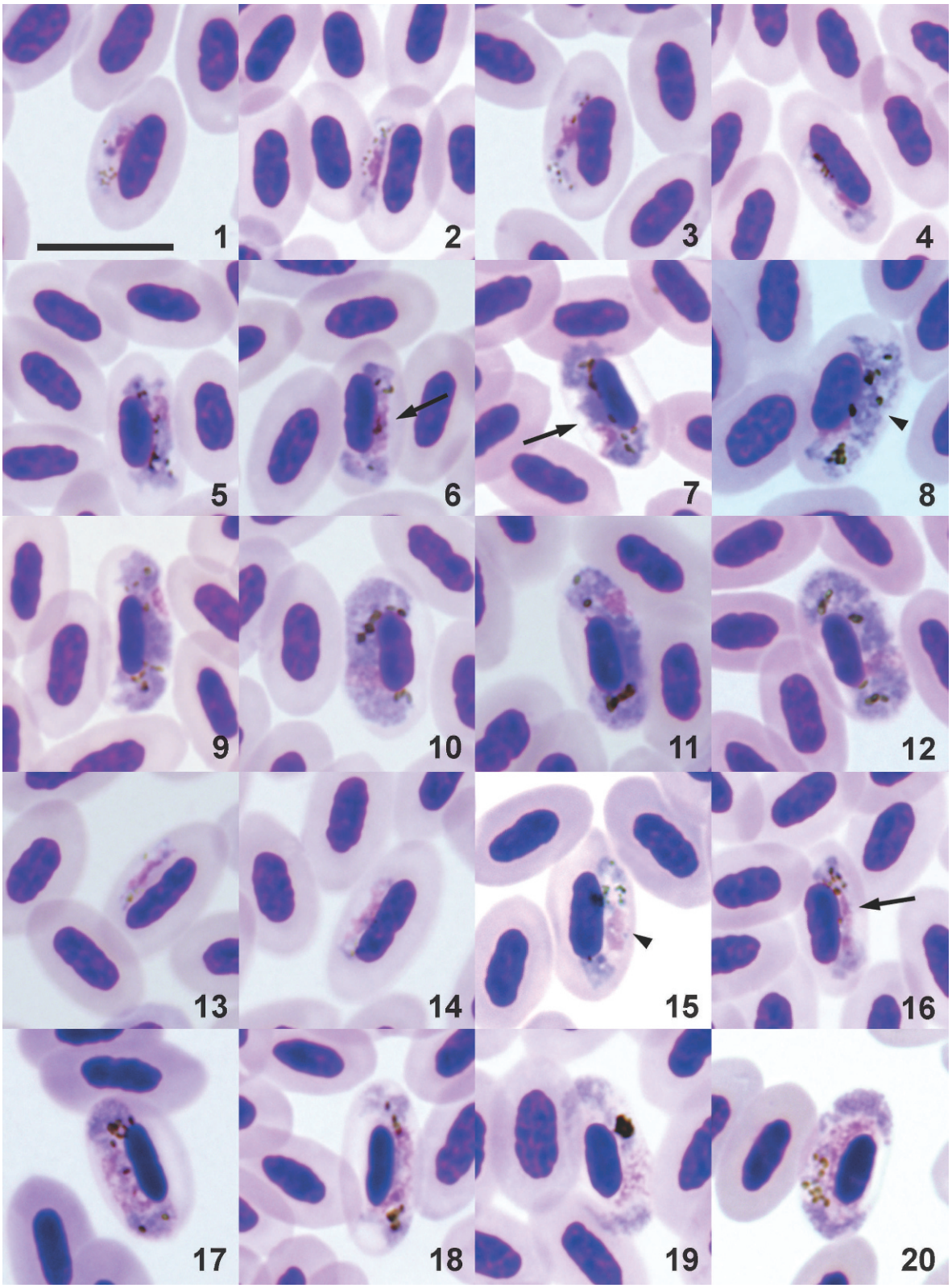
DNA sequences: Mitochondrial cyt *b* lineage HV2L (749 bp, GenBank accession FJ404696).

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

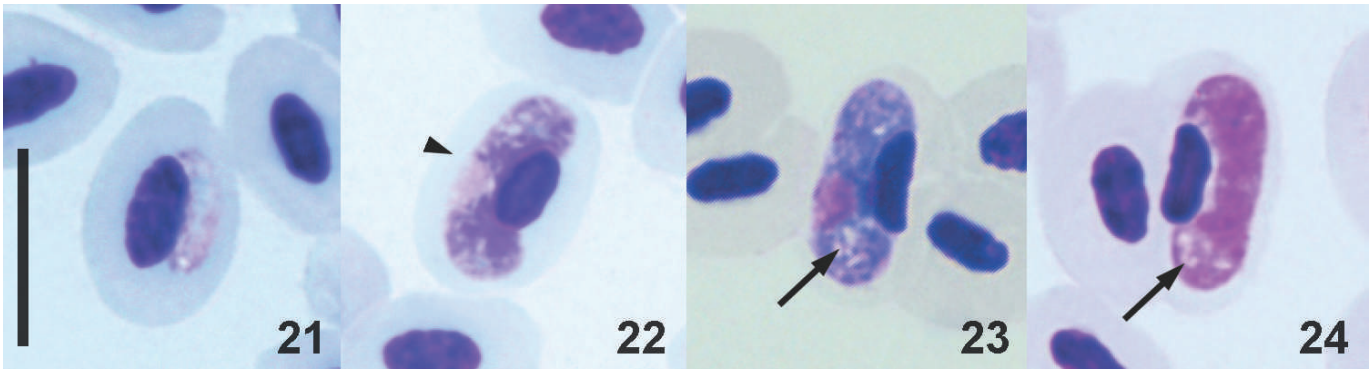
Site of infection: Mature erythrocytes; no other data.

Prevalence: Overall prevalence in the olive sunbird was 48 of 449 (10.7%). In the type locality, the prevalence was 7 of 33 (21.2%).

Distribution: According to our study and the GenBank data, this morphospecies and its lineage HV2L have been recorded in Cameroon, Ghana, Kenya, and Tanzania. The closely related lineages HV1L and HV4L (Fig. 25) have been found in the olive sunbird in Cameroon, Gabon, Ghana, Malawi, and Tanzania. It is probable that *H. cyanomitrae* is transmitted throughout the range of the olive sunbird in Africa.



FIGURES 1–20. *Haemoproteus* (*Parahaemoproteus*) *cyanomitrae* sp. nov. (lineage HV2L) from the blood of the olive sunbird, *Cyanomitra olivacea*. (**1–4**) Young gametocytes. (**5–12**) Macrogametocytes. (**13–20**) Microgametocytes. Arrows, dumbbell-shaped gametocytes. Arrowheads, unfilled space between parasites and envelope of infected erythrocytes. Giemsa-stained thin blood films. Bar = 10 μm.



FIGURES 21–24. *Haemoproteus* (*Parahaemoproteus*) *sequeirae* from the blood of the plain-throated sunbird, *Anthrepetes malacensis*. (**21**) Young gametocyte. (**22**, **23**) Macrogametocytes. (**24**) Microgametocyte. Arrows, pigment granules, which are fading in the type material of *H. sequeirae*, so they look as the colorless spots. Arrowhead, unfilled space between parasite and envelope of infected erythrocyte. Giemsa-stained thin blood films. Bar = 10 μm.

Type specimens: Hapantotype (accession 41698 NS, intensity of parasitemia is 0.7%, *Cyanomitra olivacea*, Agumatsa, Ghana, collected by G. Valkiūnas, 8 July 2007) is deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania. Parahapantotypes (accessions 47715–47720 NS, USNPC 101955, and G465372) are deposited in the Institute of Ecology, Vilnius University; in the U.S. National Parasite Collection, Beltsville, Maryland; and in the Queensland Museum, Queensland, Australia, respectively.

Etymology: The species name is derived from the genus name *Cyanomitra*, to which the type host of the parasite belongs.

Remarks

Based on the morphology of blood stages, haemoproteids of the lineages HV1L and HV4L (see Fig. 25) are indistinguishable from the lineage HV2L of *H. cyanomitrae*. Fully grown gametocytes of *H. cyanomitrae* are similar to many other species of haemoproteids of passeriform birds, so they are not easily distinguishable from many of them at this stage of their development. *Haemoproteus cyanomitrae* can be readily distinguished from the majority of described species of avian haemoproteids, primarily due to its growing dumbbell-shaped gametocytes (Fig. 5) and advanced halteridial gametocytes (Figs. 8, 15), both of which do not touch the envelope of infected erythrocytes along their entire margin. Such a mode of growth is not characteristic of the majority of haemoproteids of passeriform birds (Valkiūnas, 2005). *Haemoproteus cyanomitrae* is particularly similar to *Haemoproteus aegithinae* (De Mello, 1935) and also might be confused with *Haemoproteus sequeirae* (Tendeiro, 1947), *Haemoproteus monarchus* (Bennett et al., 1991), and *Haemoproteus vacuolatus* (Valkiūnas, Iezhova, Loiseau et al., 2008). It is worth noting that both *H. cyanomitrae* and *H. sequeirae* parasitize birds belonging to the Nectariniidae, so should be distinguished from each other during possible mixed infections.

Haemoproteus sequeirae (Figs. 21–24) can be distinguished from *H. cyanomitrae*, primarily due to markedly evident 'clefts' between growing gametocytes and the envelopes of infected erythrocytes (compare Figs. 8 and 22), markedly variable (from terminal to median) positions of nuclei in its macrogametocytes, and the absence of dumbbell-shaped growing gametocytes.

The new species is most similar to *H. aegithinae* (see Valkiūnas, 2005). It can be distinguished from the latter species, primarily due to a smaller average number of pigment granules in fully grown gametocytes (approximately 11 both in macro- and microgametocytes, with the same parameters in *H. aegithinae* being approximately 16 and 18 in macro- and microgametocytes, respectively; $P < 0.01$ for both types of gametocytes), as well as numerous oval, medium-sized (0.5–0.1-μm) pigment granules in mature gametocytes, which are absent from gametocytes of *H. aegithinae*.

Nuclei are terminal or close to terminal in position in macrogametocytes of *H. monarchus*, which is not the case with *H. cyanomitrae*. Dumbbell-shaped gametocytes were not seen in *H. vacuolatus*. In addition, a clear distinct vacuole is present in each macrogametocyte of this parasite (Valkiūnas, Iezhova, Loiseau et al., 2008); this feature is not characteristic of *H. cyanomitrae*.

Phylogenetic relationships of parasites

The majority of positively identified species of avian haemoproteids are clearly distinguishable in the phylogenetic tree (Fig. 25), which is in accord with their morphological differences. Because parasites of the lineages HV1L and HV4L, on one hand, and the lineage HV2L of *H. cyanomitrae*, on the other hand, are closely related (Fig. 25, clade a) and are indistinguishable based on morphology of their blood stages, we consider these 3 lineages as intraspecies genetic variation of the same morphospecies, *H. cyanomitrae*.

Genetic distance in cyt *b* gene between the lineages HV1L and HV4L of *H. cyanomitrae* is 0.2%, and it varies between 2.8 and 3.0% between HV2L and the former 2 lineages (Table II). Genetic differences between the lineage HV2L and lineages of other positively identified species of *Haemoproteus* are >5% (Table II).

DISCUSSION

The olive sunbird is widespread in African rain forests and serves as a convenient model organism in ecological and evolutionary biology studies for blood parasite infections (Bonneaud et al., 2009). The present study shows that *H. cyanomitrae*, the widespread haemoproteid of the olive sunbird and, to date, has been recorded only in this host. It remains unclear whether *H. cyanomitrae* parasitize other species of Nectariniidae; additional studies are needed to answer this question.

In a phylogenetic analysis (Fig. 25), we used positively identified morphospecies of avian hemoproteids, which lineages are transmitted in Africa or have been recorded in the Palearctic birds wintering in Africa, i.e., the range of transmission of *H. cyanomitrae*. Genetic distances between the cyt *b* lineage HV2L of *H. cyanomitrae* and of other readily distinguishable morphospecies of *Haemoproteus* are >5% (Table II). This is in accordance with the hypothesis of Hellgren et al. (2007), in which haemosporidian species with a genetic distance greater than 5% in the mitochondrial cyt *b* gene are expected to be morphologically differentiated. This hypothesis has been recently supported for many species of avian *Haemoproteus* and *Plasmodium* (Valkiūnas, Iezhova, Loiseau et al., 2008; Valkiūnas et al., 2009). However, there are exceptions to this rule (Hellgren et al., 2007; Valkiūnas et al., 2009); thus, the molecular criterion of >5% sequence divergence in cyt *b* gene for identification of haemosporidian species should be applied only with the careful linking of molecular and microscopy data. Accumulation of

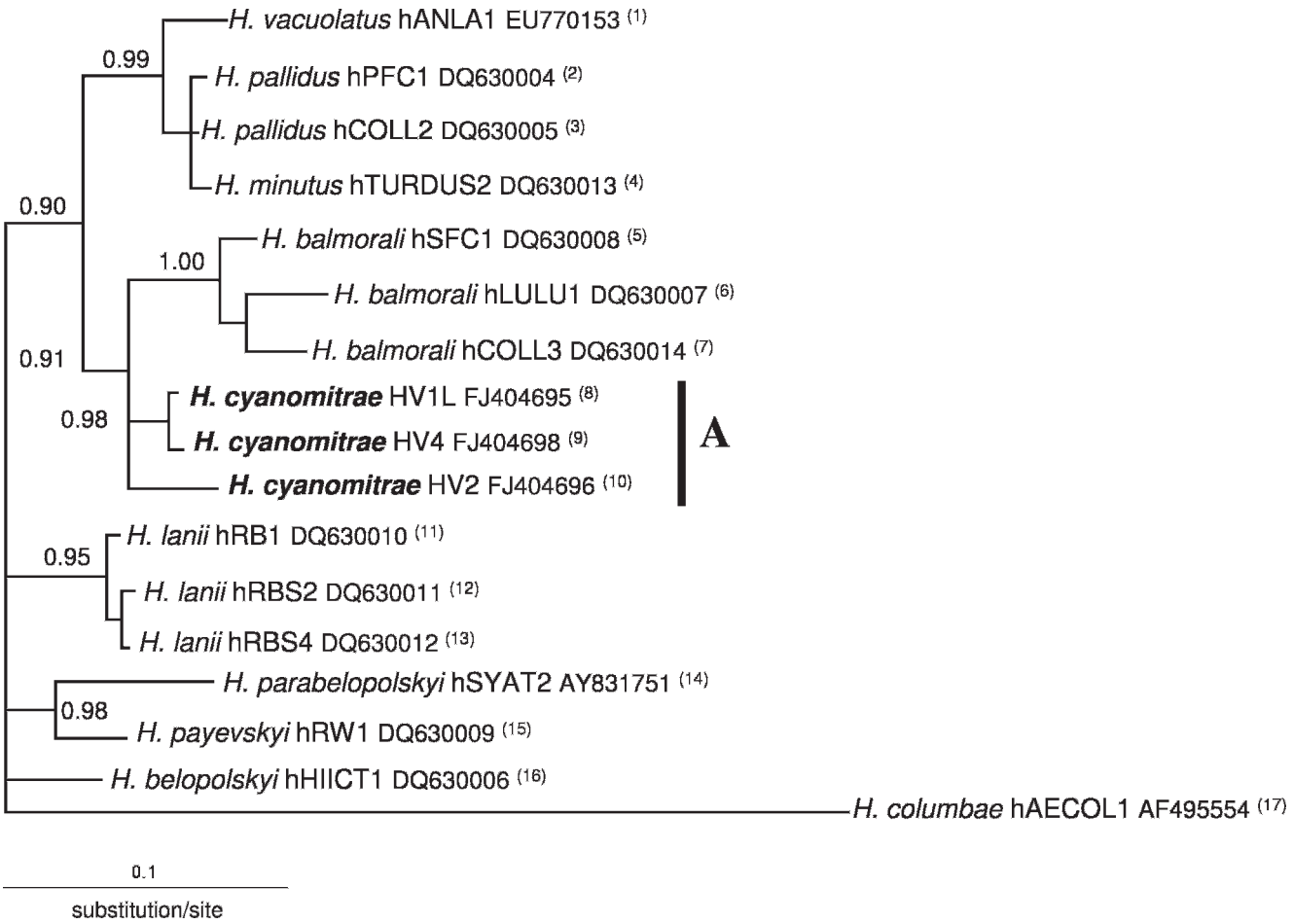


FIGURE 25. Bayesian phylogeny of 16 mitochondrial cytochrome *b* lineages of *Haemoproteus* spp. and one lineage of *Haemoproteus columbae* used as an outgroup. Posterior probabilities are indicated near the nodes. Names of the lineages and GenBank accessions of the sequences are given after the parasite species names. The lineages are numbered (ciphers in parentheses) as in Table II. Vertical bar A indicates a group of closely related lineages of new species. The name of new species is given in bold.

information on this subject is helpful for the interpretation of cyt *b* gene phylogenetic trees of avian haemosporidians and in the taxonomy of these parasites by using molecular data.

Haemoproteus cyanomitrae is tentatively associated with the subgenus *Parahaemoproteus* because its cyt *b* lineages cluster well with the lineages of other avian species of the subgenus *Parahaemoproteus* but not to the lineage of *H. columbae* (Fig. 25), which belongs to the subgenus *Haemoproteus*. Haemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of vectors (species of Hippoboscidae and Ceratopogonidae, respectively), and they undergo markedly different sporogony in the vectors (Garnham, 1966; Valkiūnas, 2005). It should be noted, however, that it is still unclear whether the phylogenetic analysis of cyt *b* genes can be applied for molecular identification of subgeneric position of all haemoproteid species. This is mainly because 1 species of the subgenus *Haemoproteus*, i.e., *H. columbae*, usually has been used in recent molecular phylogenies of haemosporidians. The position of the majority of other species of the subgenus *Haemoproteus* in the phylogenetic trees remained unknown. It worth noting that *Haemoproteus* (*Haemoproteus*) *turtur*, a common parasite of doves, appeared in the *Parahaemoproteus* clade in a 3-genome phylogeny of avian haemosporidians (Martinsen et al., 2008). Because this parasite completes sporogony in hippoboscid flies (Rashdan, 1998), it may be that molecular identification of hippoboscid-transmitted haemoproteids by using currently applied molecular markers cannot be applied to all species of hemoproteids. Sequences of other positively identified haemoproteids, which are transmitted by hippoboscids, are needed to clarify this issue. Thus, further work to increase the number of precise linkages between haemosporidian DNA lineages with their morphospecies, particularly of hippoboscid-transmitted species of the subgenus *Haemoproteus*, is an important task.

It worth noting that fading of blood stages of hemosporidian parasites, including fading of pigment granules, is a common phenomenon in relatively old blood films (>10 yr old), which were stained with Rapid stains, for example, Field's stain (see Valkiūnas, 2005). Unfortunately, that is a case with the type material of *H. sequeirae* (Figs. 21–24). Staining with Giemsa is more stable in this respect, so is more suitable for taxonomic investigations. For example, C. M. Wenyon's samples of blood parasites, which are available in the Natural History Museum (London) and were stained with Giemsa approximately 100 yr ago, are still in excellent condition (G. Valkiūnas, unpubl. obs.). This should be considered during sampling and preparation of material for investigation of blood parasites.

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