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NEW AVIAN *HAEMOPROTEUS* SPECIES (HAEMOSPORIDA: HAEMOPROTEIDAE) FROM AFRICAN BIRDS, WITH A CRITIQUE OF THE USE OF HOST TAXONOMIC INFORMATION IN HEMOPROTEID CLASSIFICATION

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ABSTRACT: *Haemoproteus* (*Parahaemoproteus*) *micronuclearis* n. sp., *Haemoproteus* (*Parahaemoproteus*) *nucleofascialis* n. sp., *Haemoproteus* (*Parahaemoproteus*) *paranucleophilus* n. sp., and *Haemoproteus* (*Parahaemoproteus*) *homobelopolskyi* n. sp. (Haemosporida, Haemoproteidae) are described from African passeriform birds based on the morphology of their blood stages and segments of the mitochondrial cytochrome *b* gene. Red-billed quelea (*Quelea quelea*), red-headed malimbe (*Malimbus rubricollis*), and black-headed weaver (*Ploceus melanocephalus*) are the type vertebrate hosts of new hemoproteids. It is probable that new species have wide distribution in weavers in sub-Saharan Africa. Both *H. micronuclearis* and *H. nucleofascialis* can be readily distinguished from other avian hemoproteids by tiny, compact microgametocyte nuclei that are significantly smaller than macrogametocyte nuclei and are a rare character of hemosporidian parasites. Gametocytes of *H. paranucleophilus* are closely appressed to the erythrocyte nuclei and do not touch the erythrocyte envelope along their entire margin at all stages of their development, including fully grown gametocytes. A particularly distinctive feature of *H. homobelopolskyi* development is the presence of circumnuclear dumbbell-shaped macrogametocytes. Illustrations of blood stages of the new species are given, and morphological and phylogenetic analyses identify the DNA lineages that are associated with these parasites. Numerous recent studies show that some lineages of hemoproteids are often present in birds belonging to different families. As a result, the use of the host family as a taxonomic character should be questioned and preferably discouraged in hemoproteid taxonomy, particularly with regard to the parasites of passerine birds. Microscopic identification of avian hemoproteids requires comparison of *Haemoproteus* species described from birds of different families, as is an established practice with avian *Plasmodium* spp. Development of bar-coding techniques remains essential in taxonomic and field studies of hemosporidian parasites.

Species of *Haemoproteus* (Haemosporida, Haemoproteidae) are widespread and abundant dipteran-borne hemosporidian parasites (Haemosporida) and have been reported in birds on all continents except Antarctica (Garnham, 1966; Bishop and Bennett, 1992). More than 140 species of avian hemoproteids have been described previously (Valkiūnas, 2005; Iezhova et al., 2010). The majority of these species belong to the subgenus *Parahaemoproteus* and are transmitted by biting midges belonging to *Culicoides* (Ceratopogonidae). Currently, only 7 species have been assigned to the subgenus *Haemoproteus*, all of which are transmitted by hippoboscid flies (Hippoboscidae; Bennett et al., 1965; Garnham, 1966; Valkiūnas, Santiago-Alarcon et al., 2010). Hemoproteids are often considered to be relatively benign to their avian hosts and vectors (Bennett et al., 1993). However, some species cause severe pathology in birds (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002), are sometimes even lethal (Ferrell et al., 2007), and can affect host fitness (Nordling et al., 1998; Marzal et al., 2005; Valkiūnas, 2005; Møller and Nielsen, 2007). Heavy hemoproteid infections are also pathogenic and sometimes even kill biting midges (Valkiūnas and Iezhova, 2004). This phenomenon has not been sufficiently investigated in wildlife. Characterization of the distribution and diversity of avian hemoproteids is important to better understand the challenges facing wildlife health.

Recent molecular studies have revealed extraordinary genetic diversity of avian hemosporidian parasites in terms of speciation. This indicates that the number of hemosporidian species is probably much greater than have been described to date (Perkins and Schall, 2002; Bensch et al., 2004, 2009; Ricklefs et al., 2004). However, development of hemosporidian taxonomy on a species level is a difficult task because it requires morphological information about main stages of parasites' development in the blood (immature, growing, and mature gametocytes in the hemoproteids). It is essential to describe these developmental stages to make morphological comparisons between potential new species and already described parasites. Such stages are rarely present simultaneously in each blood sample because light parasitemias predominate in free-living birds. That is the main obstacle in identification and description of hemosporidian species based on limited sampling, typical of short-term expeditions when each individual host usually is examined only once. Blood samples containing a full range of developing blood stages remain of considerable value for taxonomic purposes. The identification of molecular markers for distinguishing hemosporidian species is an important task because it provides opportunities to detect parasite species at particularly light parasitemias, which predominate in wildlife.

Mitochondrial cytochrome *b* (cyt *b*) gene fragments have been successfully used in distinguishing hemosporidian species; they are easy to use and are convenient for taxonomic work with hemosporidian parasites (Martinsen et al., 2006; Sehgal et al., 2006; Palinauskas et al., 2007; Valkiūnas et al., 2009; Iezhova et al., 2010; Križanauskienė et al., 2010; Santiago-Alarcon et al., 2010). In fact, a public database called MalAvi has been launched to coordinate the study of avian malaria parasite diversity among research groups (Bensch et al., 2009).

Here, we describe 4 new species of avian hemoproteids from African passeriform birds. Red-billed quelea (*Quelea quelea*), red-headed malimbe (*Malimbus rubricollis*), and black-headed weaver (*Ploceus melanocephalus*) are the type vertebrate hosts of the new hemoproteids, and they probably have wide distribution in weavers in sub-Saharan Africa. We identify cyt *b* lineages that can be used for the parasites' molecular diagnostics. We also discuss recent molecular and morphological findings regarding the natural host range of avian hemoproteids. This is important

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for understanding vertebrate host specificity, which currently is still in use as a prominent taxonomic character in avian hemoproteid taxonomy and frequently relates parasites at the host family level (Barraclough et al., 2008; Parsons et al., 2010).

MATERIAL AND METHODS

Collection of blood samples

Blood samples were collected in July 2003 (the dry season) in Uganda, as described by Valkiūnas et al. (2005). Material for this study was collected in Kibale National Park and Queen Elizabeth National Park, Uganda. New species of hemoproteids were found in species of the Ploceidae; 16 individual ploceid birds of 5 species were investigated. Seven individual birds (prevalence is 44%) of 4 species (red-headed malimbe, black-headed weaver, slender-billed weaver [*Ploceus pelzelni*], and red-billed quelea) harbored hemoproteids (see Valkiūnas et al., 2005). Birds were caught with mist nets between daybreak (0600 hr) and dusk (1700 hr). They were ringed, bled, and released. None of them was recaptured. One or 2 drops of blood were taken by puncturing the brachial vein.

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.

Three blood films were prepared from each bird. Blood films were air-dried within 5–10 sec 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 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). 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 described by Godfrey et al. (1987). To determine possible presence of simultaneous infections with other hemosporidian 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 and imaging software analySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations, and to take measurements. The morphometric features studied (Table I) are those defined by Valkiūnas (2005). Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A P value of ≤ 0.05 was considered significant.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

DNA was extracted from whole blood using the Wizard SV Genomic DNA Purification System (Promega, Madison, Wisconsin). Extraction success was verified by PCR using primers that amplify the gene encoding the brain-derived neurotrophic factor (Sehgal and Lovette, 2003).

Haemoproteus spp. were detected by nested PCR amplification of a fragment of the cyt *b* region of the mitochondrial DNA following the protocol of Waldenström et al. (2004), with a few modifications. The PCR products of amplification by primers HAEMNF 5′-CATATATTAAGAGAATTATGGAG-3′ and HAEMNR2 5′-AGAGGTGTAGCATATCTATCTAC-3′ were used as the template for a secondary amplification by primers HAEMF 5′-ATGGTGCTTTCGATATATGCATG-3′ and HAEMR2 5′-GCATTATCTGGATGTGATAATGGT-3′. Each reaction included approximately 10–100 ng of genomic DNA, 2.5 mM $MgCl_2$, 5 µl of 5× GoTaq Flexi buffer, 400 µM of each deoxynucleoside triphosphates, 0.6 µM of each primer, and 0.625 U of GoTaq Flexi DNA polymerase (Promega). The thermal profile for amplification of the "outer" fragment started with 3 min of denaturation at 94 C, followed by 20 cycles at 94 C for 30 sec, 50 C for 30 sec, and 72 C for 45 sec, and ended with an elongation step at 72 C for 10 min. The second, inner fragment was amplified using the same reagents and thermal profile as described above, but for 35 cycles instead of 20. All reactions were performed in 25-µl volumes and were accompanied by negative (double distilled H_2O) and positive controls (samples from infected birds, confirmed by microscopy) to control for any contamination and to confirm success of the PCR.

PCR products were purified using ExoSAP according to the manufacturer's instructions (United States Biochemical Corporation, Cleveland, Ohio); they were sequenced to identify parasite lineages (BigDye version 1.1 sequencing kit, Applied Biosystems, Foster City, California) on an ABI Prism 3100 automated sequencer (Applied Biosystems).

Sequences were aligned using the program Sequencher 4.8 (Gene Codes, Ann Arbor, Michigan). In cases where the chromatogram revealed at least 1 double peak and microscopy showed co-infection with different morphospecies, the sample was subjected to an additional amplification using the nested PCR described above. The resulting PCR product was then cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, California) following Sambrook and Russell (2001) and the manufacturer's instructions. Ten colonies were amplified per sample using the PCR methods described above, and PCR products from each colony were sequenced to identify what lineage was present. When co-infections were present, the linking of lineages to morphospecies was performed as follows. First, we assigned lineages to morphospecies in samples containing single infections. Single infections were determined both by microscopy and PCR diagnostics as determined by the absence of "double peaks" in electropherograms of cyt *b* sequences. All co-infections were thus clearly identified, compared with lineages of single infections. The remaining unassigned lineages were linked to the second morphospecies present in samples with co-infections. We are confident about these lineage assignments because the morphology of new *Haemoproteus* species differed significantly, and we have samples with single infections and co-infections at our disposal.

We used the BLAST algorithm to compare the sequences of new lineages to known *Haemoproteus* spp. lineages deposited in GenBank.

Phylogenetic analysis

We used 53 mitochondrial cyt *b* sequences of avian *Haemoproteus* species from our survey and from GenBank. The GenBank sequences included in the phylogenetic analysis were carefully chosen to correspond to positive morphological identifications, i.e., identified by targeting taxonomic studies. For examples of parasite lineage linkage to their morphospecies and additional literature on this subject, see Križanauskienė et al. (2006), Sehgal et al. (2006), Hellgren et al. (2007), Palinauskas et al. (2007), Valkiūnas et al. (2007), Martinsen et al. (2008), Valkiūnas et al. (2008, 2009), Iezhova et al. (2010), and Valkiūnas, Santiago-Alarcon et al. (2010). Two lineages of *Leucocytozoon schoutedeni* were used as outgroups and 5 *Plasmodium* taxa also were included in genetic distance analysis. Sequences were aligned using Sequencher 4.8 and grouped into a consensus that was 487 bp.

The phylogenetic tree was created using mrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). The appropriate model of sequence evolution was determined by the software mrModeltest (Nylander, 2004) to be a General Time Reversible model including variable sites (GTR+I). The Bayesian phylogeny was constructed using 1 cold of 2 hot Monte Carlo Markov chains, which were sampled every 200 generations over 20 million generations for a total of 100,000 generated trees. In all, 25% of these were discarded as burn in, and the remaining 75,000 trees were used to construct a majority consensus tree.

The sequence divergence among lineages was calculated using a Jukes–Cantor model of substitution in which all substitutions were weighted equally (PAUP 4.0; Swofford, 2003).

DESCRIPTION

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

(Figs. 1–16; Table I)

Diagnosis: Young gametocytes: Earliest forms were not seen in the type material.

Macrogametocytes (Figs. 1–8): Gametocytes grow along nuclei of erythrocytes, displacing nuclei laterally; closely associated with both nuclei and envelope of erythrocytes. Growing gametocytes do not fill erythrocytes up to their poles (Figs. 2, 3), a characteristic feature of this species' development. Fully grown gametocytes halteridial, slightly enclosing erythrocyte nuclei with their ends and displacing them laterally, but do not encircle nuclei completely; fill erythrocytes up to their poles (Figs. 4–8).

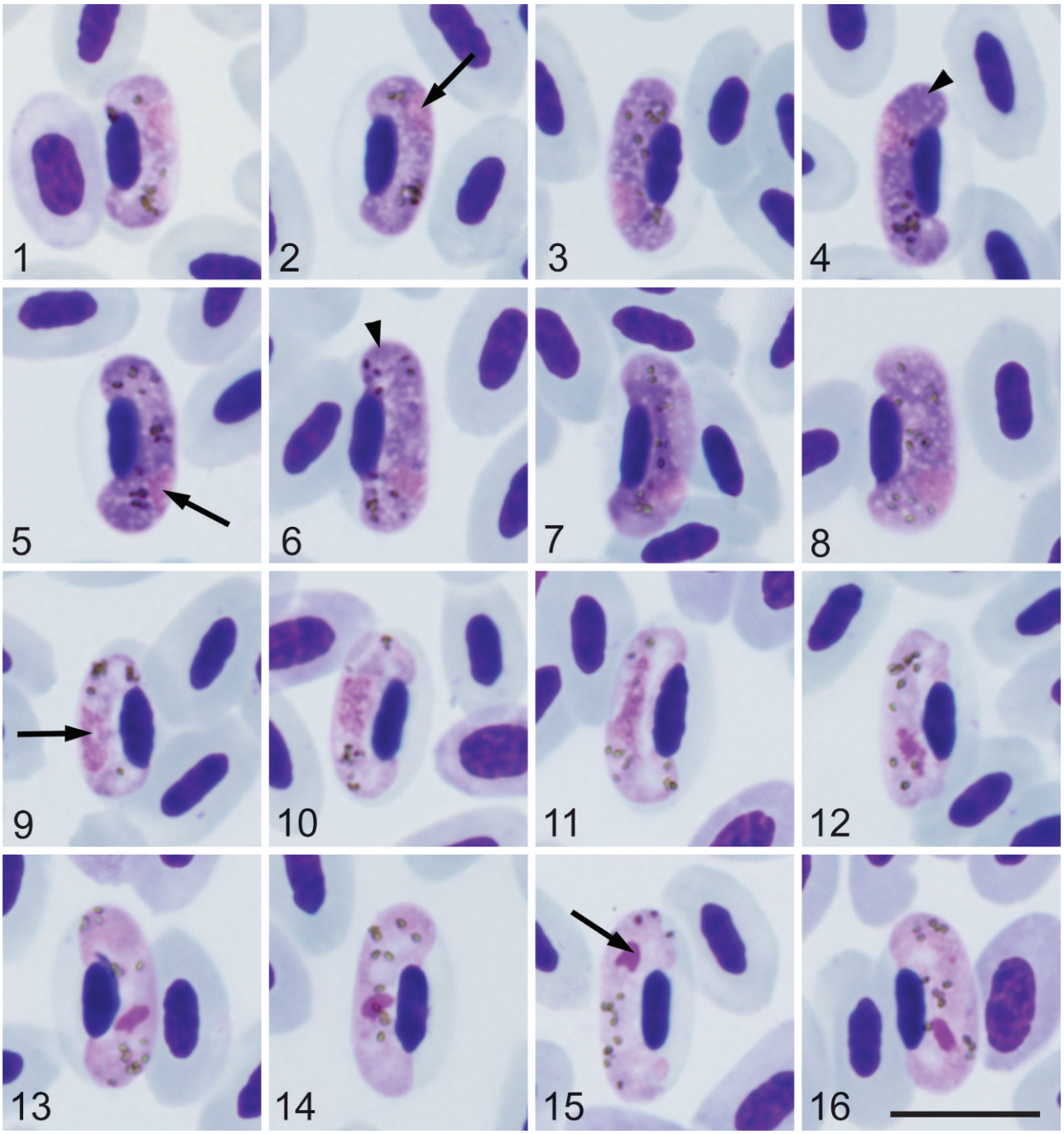
TABLE I. Morphometry of host cells and mature gametocytes of 4 new species of *Haemoproteus* from African birds.

Feature	Measurements*			
	H. micronuclearis	*H. nucleofascialis*	*H. paranucleophilus*	*H. homobelopolskyi*
Uninfected erythrocyte				
Length	10.4–11.9 (11.3 ± 0.5)	10.4–12.2 (11.5 ± 0.5)	10.4–12.2 (11.5 ± 0.5)	9.8–11.5 (10.5 ± 0.5)
Width	5.4–6.6 (6.0 ± 0.3)	6.1–7.4 (6.7 ± 0.4)	6.1–7.4 (6.7 ± 0.4)	6.0–7.2 (6.5 ± 0.3)
Area	48.7–65.0 (55.7 ± 4.0)	51.3–70.2 (61.2 ± 5.3)	51.3–70.2 (61.2 ± 5.3)	45.4–62.5 (53.8 ± 4.4)
Uninfected erythrocyte nucleus				
Length	4.2–5.7 (5.1 ± 0.4)	4.7–6.0 (5.3 ± 0.3)	4.7–6.0 (5.3 ± 0.3)	4.3–5.4 (4.8 ± 0.3)
Width	1.7–2.3 (2.0 ± 0.2)	2.0–2.5 (2.2 ± 0.2)	2.0–2.5 (2.2 ± 0.2)	2.0–2.7 (2.4 ± 0.2)
Area	6.7–10.6 (9.0 ± 1.0)	8.9–13.1 (10.3 ± 1.0)	8.9–13.1 (10.3 ± 1.0)	8.7–10.6 (9.6 ± 0.5)
Macrogametocyte				
Infected erythrocyte				
Length	11.9–13.2 (12.4 ± 0.3)	11.0–13.0 (11.9 ± 0.4)	11.8–13.7 (12.8 ± 0.5)	10.2–12.7 (11.7 ± 0.5)
Width	5.5–7.0 (6.3 ± 0.4)	6.3–7.9 (6.9 ± 0.4)	5.7–7.2 (6.4 ± 0.5)	5.3–7.2 (6.6 ± 0.5)
Area	52.3–69.7 (62.9 ± 4.5)	57.7–72.9 (64.9 ± 3.9)	56.4–76.0 (67.5 ± 5.7)	43.3–70.0 (62.1 ± 5.4)
Infected erythrocyte nucleus				
Length	5.1–5.9 (5.4 ± 0.2)	4.8–6.0 (5.4 ± 0.4)	3.8–6.2 (5.0 ± 0.5)	4.0–5.7 (5.0 ± 0.4)
Width	1.6–2.0 (1.8 ± 0.1)	1.8–2.6 (2.2 ± 0.2)	1.8–2.5 (2.2 ± 0.2)	1.5–2.4 (2.0 ± 0.2)
Area	7.8–10.0 (8.7 ± 0.5)	8.4–11.4 (10.0 ± 0.8)	8.1–11.7 (9.7 ± 0.9)	7.4–10.0 (9.0 ± 0.7)
Gametocyte				
Length	12.4–14.1 (13.4 ± 0.4)	10.2–12.1 (11.1 ± 0.5)	11.6–13.6 (12.6 ± 0.7)	15.5–19.1 (17.0 ± 1.1)
Width	2.0–3.5 (2.7 ± 0.3)	2.3–4.0 (3.2 ± 0.4)	0.8–1.4 (1.1 ± 0.2)	1.4–3.8 (2.4 ± 0.5)
Area	34.2–49.0 (40.6 ± 4.0)	24.1–40.3 (34.0 ± 4.3)	14.9–20.2 (17.6 ± 1.4)	36.5–49.7 (43.0 ± 3.9)
Gametocyte nucleus				
Length	2.1–3.8 (3.2 ± 0.3)	1.9–3.2 (2.5 ± 0.4)	1.8–3.2 (2.5 ± 0.4)	2.1–3.6 (2.7 ± 0.4)
Width	1.2–2.8 (2.0 ± 0.4)	1.1–2.1 (1.7 ± 0.3)	1.1–2.2 (1.5 ± 0.3)	1.5–2.2 (1.9 ± 0.2)
Area	3.3–6.1 (4.6 ± 0.7)	1.9–4.6 (3.2 ± 0.7)	1.6–4.2 (3.1 ± 0.6)	3.0–4.7 (3.8 ± 0.4)
Pigment granules	8.0–16.0 (13.0 ± 2.0)	8.0–12.0 (10.0 ± 1.2)	9.0–13.0 (10.2 ± 1.2)	12.0–18.0 (14.6 ± 1.4)
NDR†	0.5–1.0 (0.7 ± 0.1)	0.4–0.9 (0.6 ± 0.1)	0.7–1.2 (0.9 ± 0.1)	0.4–0.9 (0.7 ± 0.1)
Microgametocyte				
Infected erythrocyte				
Length	11.7–13.6 (12.4 ± 0.4)	11.3–13.5 (12.3 ± 0.6)	12.1–14.1 (13.2 ± 0.6)	11.0–13.0 (12.0 ± 0.5)
Width	6.0–7.4 (6.6 ± 0.4)	5.8–7.3 (6.7 ± 0.3)	5.5–7.1 (6.4 ± 0.4)	6.1–7.6 (6.9 ± 0.4)
Area	56.8–73.7 (66.0 ± 4.2)	55.4–71.9 (65.6 ± 4.3)	56.4–80.2 (69.0 ± 5.4)	52.9–72.1 (64.8 ± 4.7)
Infected erythrocyte nucleus				
Length	4.8–6.0 (5.3 ± 0.3)	4.6–6.4 (5.5 ± 0.4)	4.3–5.6 (4.9 ± 0.3)	4.2–6.0 (5.0 ± 0.4)
Width	1.5–2.4 (1.7 ± 0.2)	1.9–2.5 (2.2 ± 0.2)	1.9–2.4 (2.2 ± 0.1)	1.5–2.4 (2.0 ± 0.2)
Area	6.5–9.3 (8.1 ± 0.8)	9.2–12.0 (10.4 ± 0.8)	7.5–11.4 (9.3 ± 0.9)	7.1–9.9 (8.5 ± 0.7)
Gametocyte				
Length	12.2–13.8 (12.9 ± 0.5)	11.0–13.8 (12.3 ± 0.7)	13.7–15.7 (14.9 ± 0.5)	14.5–18.9 (16.6 ± 1.1)
Width	2.5–3.6 (3.0 ± 0.3)	2.3–4.0 (3.0 ± 0.4)	0.9–1.8 (1.3 ± 0.3)	2.2–3.1 (2.8 ± 0.2)
Area	36.1–48.9 (42.4 ± 3.6)	32.0–42.2 (36.8 ± 2.8)	20.5–27.6 (23.8 ± 1.6)	31.9–52.4 (44.1 ± 4.5)
Gametocyte nucleus				
Length	1.4–3.3 (2.3 ± 0.4)	2.2–6.3 (4.5 ± 1.0)	—	7.7–12.4 (9.6 ± 1.3)
Width	0.8–1.8 (1.1 ± 0.2)	0.5–1.3 (0.8 ± 0.2)	—	1.3–2.7 (2.2 ± 0.3)
Area	1.6–2.9 (2.2 ± 0.4)	1.8–3.3 (2.5 ± 0.4)	—	10.2–22.1 (15.0 ± 3.3)
Pigment granules‡	10.0–16.0 (12.7 ± 1.9)	3.0–11.0 (7.9 ± 2.0)	—	13.0–22.0 (17.5 ± 2.8)
NDR	0.6–0.9 (0.7 ± 0.1)	0.2–1.0 (0.6 ± 0.2)	0.7–1.2 (1.0 ± 0.1)	0.7–1.0 (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 SD.
† NDR = nucleus displacement ration according to Bennett and Campbell (1972).
‡ Because of aggregation of pigment granules in prominent masses, their number was not calculated in microgametocytes of *H. paranucleophilus.*

Parasite nucleus compact, variable in shape, but more often oval, and subterminal in position (Figs. 2–8). Nucleolus not observed. Pigment granules roundish or slightly oval and of medium size (0.5–1 μm). Mostly randomly scattered throughout cytoplasm but sometimes grouped (Figs. 2, 4, 5). In majority of gametocytes, they are of sister-size and shape, a characteristic feature of this species. Outline of gametocytes even. Cytoplasm blue, homogeneous in appearance, usually possess small (<0.5-μm) vacuoles; volutin present and looks dark violet, with prominent clumps irregularly scattered throughout cytoplasm.

Microgametocytes (Figs. 9–16): General configuration as for macrogametocytes, with main hemosporidian sexually dimorphic characters. Parasite nuclei diffuse in growing gametocytes (Figs. 9–11); begin to compress in advanced gametocytes (Fig. 12) and then markedly compressed in fully grown gametocytes. Nuclei of fully grown gametocytes include distinct red



FIGURES 1–16. *Haemoproteus* (*Parahaemoproteus*) *micronuclearis* sp. nov. from the blood of red-billed quelea (*Quelea quelea*). (**1–8**) Macrogametocytes. (**9–16**) Microgametocytes. Long arrows, nuclei of parasites. Arrowheads, clamps of volutin. Giemsa-stained thin blood films. Bar = 10 μm.

spots of variable shape (Figs. 13–16) but never assume band-like forms; not associated with either the nuclei or envelope of erythrocytes (Figs. 13–16). Length, width, and area of nuclei of fully grown microgametocytes (Figs. 13–16) significantly less than same parameters of macrogametocyte nuclei (Table I; $P < 0.001$ for each feature). Sister-size and sister-shape of pigment granules clearly evident in fully grown gametocytes (Figs. 13–16).

Taxonomic summary

Type host: Red-billed quelea (*Quelea quelea* L. [Passeriformes, Ploceidae]).

Type locality: Queen Elizabeth National Park (0°17.8′S, 30°3.0′E, 1,000 m above sea level) Uganda.

Type specimens: Hapantotype (accession 6916 NS, intensity of parasitemia is approximately 0.1%, lineage HV36, GenBank HQ386235, *Q. quelea*, Queen Elizabeth National Park, Uganda, 0°17.8′S, 30°3.0′E, collected by G. Valkiūnas, 19 July 2003) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accessions 6917 NS and G465455) are deposited in the Institute of Ecology, Nature Research Centre, and in the Queensland Museum, Queensland, Australia, respectively. Fully grown gametocytes are marked by circles on the hapantotype and parahapantotype slides.

Additional material: Two blood films (accessions G465457 and G465459, intensity of parasitemia is 1%, *Ploceus melanocephalus*, Queen Elizabeth National Park, 0°17.8′S, 30°3.0′E, lineage HV37, collected by G. Valkiūnas, 19 July 2003) are deposited in the Queensland Museum.

DNA sequences: Mitochondrial cyt *b* lineages HV36, HV37, HV38, HV39, and HV40, (GenBank HQ386235, HQ386236, HQ386237, HQ386238, and HQ386239, respectively).

Site of infection: Mature erythrocytes; no other data.

Prevalence: One of 1 investigated red-billed quelea was infected at the type locality.

Distribution and additional hosts: According to this study and GenBank data, *H. micronuclearis* was recorded in 1 black-headed weaver (lineage GenBank HQ386236) in the type locality and 1 Vieillot's weaver (*Ploceus nigerrimus*) in Cameroon (HQ386237, HQ386238, and HQ386239). Closely related lineage WAH33 (EU810735; genetic distance from all lineages of *H. micronuclearis* is <0.01%) was recorded in red-headed quelea (*Quelea erythrops*) in Gabon (Beadell et al., 2009). It is probable that *H. micronuclearis* is widespread in sub-Saharan Africa. Transmission of the parasite certainly occurs among birds belonging to *Quelea* and *Ploceus*; other hosts are unknown.

Etymology: The species name reflects the small size and compactness of nuclei in fully grown microgametocytes (Figs. 13–16); the most distinctive feature of *H. micronuclearis*.

Remarks

Macrogametocytes of *H. micronuclearis* are similar to macrogametocytes of many other species of avian hemoproteids (Valkiūnas, 2005), so this parasite can hardly be distinguished solely on morphology of these blood stages. However, it can be readily distinguished from other species of avian hemoproteids by the tiny, compact microgametocyte nuclei; they are approximately half the size of nuclei in macrogametocytes (Table I). Such compact microgametocyte nuclei are a rare character for hemosporidian parasites. Among avian hemoproteids, the compression of nuclear material in mature microgametocytes has been reported only in *Haemoproteus payevskyi*, which probably is only transmitted in Africa (Valkiūnas, 2005). The latter species can be readily distinguished from *H. micronuclearis* by the median position of nuclei both in macro- and microgametocytes, and the grouping of pigment granules into loose clumps located close to the ends of gametocytes. Genetic divergence among different lineages of *H. micronuclearis* and *H. payevskyi* varies between 5.5 and 6%.

Haemoproteus (*Parahaemoproteus*) *nucleofascialis* n. sp.
(Figs. 17–32; Table I)

Diagnosis: Young gametocytes (Figs. 17–19): Develop in mature erythrocytes. Earliest forms not observed in type material. With development (size greater than erythrocyte nuclei), gametocytes closely associate with nuclei of infected erythrocytes. They do not touch envelope of erythrocytes along entire margin (Figs. 17–19), a characteristic feature of this species' development. Parasite nucleus prominent, sub-terminal (Fig. 18) or terminal (Figs. 17, 19) in position. Pigment granules medium size (0.5–1 μm), roundish or slightly oval, dark brown or black, and frequently grouped (Figs. 18, 19). Outline even and volutin granules present. Growing gametocytes displace erythrocyte nuclei laterally (Figs. 17–19).

Macrogametocytes (Figs. 20–24): Gametocytes grow along nuclei of erythrocytes, displacing nuclei laterally; closely associated with both nucleus and envelope of erythrocytes. Growing gametocytes slightly enclose erythrocyte nuclei with their ends; do not fill erythrocytes up to their poles (Figs. 20–23), but fully grown gametocytes occupy all, or nearly all, cytoplasmic space on poles of erythrocytes (see Fig. 24 and also microgametocyte in Fig. 32). Fully grown gametocytes halteridial, and only slightly enclose erythrocyte nuclei with their ends, but they markedly displace them laterally (Fig. 24). Parasite nucleus compact, markedly variable in shape, and sub-terminal (Figs. 20, 21, 23, 24) or terminal (Fig. 22) in position. Nucleolus not observed. Pigment granules roundish or slightly oval, dark brown, of medium size (0.5–1 μm), usually randomly scattered throughout cytoplasm (Figs. 20, 23, 24), although sometimes grouped (Figs. 21, 22). Individual granules markedly variable in size (non-sister-size). Outline of gametocytes even (Figs. 23, 24) or slightly angular (Figs. 20–22). Cytoplasm blue, homogeneous in appearance, usually lacks visible vacuoles, and possesses dark violet volutin granules.

Microgametocytes (Figs. 25–32): General configuration as for macrogametocytes, with main hemosporidian sexually dimorphic characters. Parasite nucleus diffuse in growing gametocytes (Fig. 25). Nuclei begin to compress as parasite matures (Fig. 26). In fully grown gametocytes, nuclei markedly compressed, and usually assume band-like shape, closely associated with parasite pellicle located close to erythrocyte envelope (Figs. 27–31); these 2 attributes of gametocyte nuclei distinctive morphological characters of this species. Occasionally, nuclei assume irregular shape but are still closely associated with gametocyte pellicle close to erythrocyte envelope (Fig. 28). Area of microgametocyte nuclei significantly less than that of macrogametocytes (Table I; $P < 0.001$), a rare character for bird hemosporidian parasites.

Taxonomic summary

Type host: Red-headed malimbe (*Malimbus rubricollis* L. [Passeriformes, Ploceidae]).

Type locality: Kibale National Park (0°34.7′N, 30°21.3′E, 1,580 m above sea level), Uganda.

Type specimens: Hapantotype (accession 6465 NS, intensity of parasitemia is approximately 0.01%, lineage HV44, GenBank HQ386243, *M. rubricollis*, Kibale National Park, Uganda, collected by G. Valkiūnas, 12 July 2003) and parahapantotype (accession 6463 NS) are deposited in the Institute of Ecology, Nature Research Centre. Fully grown gametocytes are marked by circles on the hapantotype and parahapantotype slides.

DNA sequences: Mitochondrial cyt *b* lineages HV44 and HV45 (GenBank HQ386243 and HQ386244, respectively).

Site of infection: Mature erythrocytes; no other data.

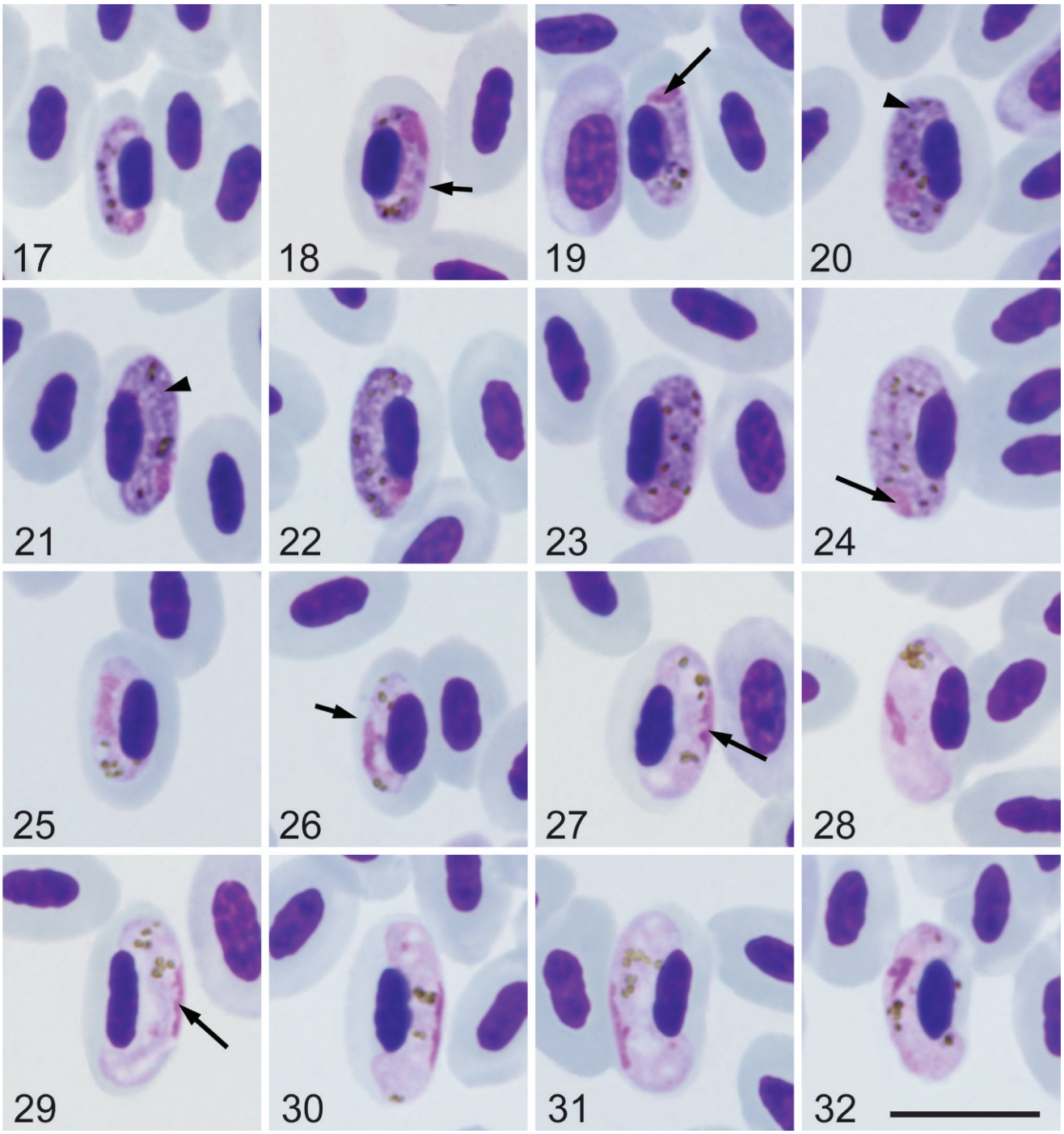
Prevalence: One of 1 investigated red-headed malimbe was infected in the type locality.

Distribution and additional hosts: According to our study and the GenBank data, the lineage HV45 (GenBank HQ386244) was recorded in 1 black-headed weaver in the Queen Elizabeth National Park. A closely related lineage WAH11 (EU810731, genetic distance between WAH11 and 2 lineages of *H. nucleofascialis* is between 0.021 and 0.004%) was recorded in Gray's malimbe (*Malimbus nitens*) in Gabon (Beadell et al., 2009). It is probable that *H. nucleofascialis* is widespread in sub-Saharan Africa. Transmission of the parasite certainly occurs among birds belonging to species of *Ploceus* and *Malimbus*.

Etymology: The species name reflects the compact, band-like shape of nuclei in fully grown microgametocytes. This is the most distinctive character of *H. nucleofascialis*.

Remarks

Haemoproteus nucleofascialis should be distinguished from avian hemoproteid species that possess halteridial gametocytes by the following features of their growth: (1) the growing gametocytes (size greater than erythrocyte nuclei) are closely appressed to erythrocyte nuclei but do not touch the erythrocyte envelope along their entire margin (Figs. 17, 18) and (2) the fully grown gametocytes are closely appressed both to nuclei and envelope of erythrocytes (Figs. 23, 24). Twenty-four hemoproteid species with such gametocytes are known to parasitize birds (see Valkiūnas, 2005; Valkiūnas et al., 2008; Iezhova et al., 2010): *H. aegithinae*, *H. africanus*, *H. attenuatus*, *H. bubalornis*, *H. buteonis*, *H. coatneyi*, *H. cyanomitrae*, *H. eurystomae*, *H. formicarius*, *H. kairullaevi*, *H. manwelli*, *H. minutus*, *H. monarchus*, *H. neseri*, *H. otocompsae*, *H. pachycephalus*, *H. porzanae*, *H. quiscalus*, *H. sanguinis*, *H. sequeirae*, *H. tyranni*, *H. vacuolatus*, *H. vireonis*, and *H. xantholaemae*. *Haemoproteus nucleofascialis* can be readily distinguished from these parasites due to its band-like, and markedly compressed, microgametocyte nuclei. These nuclei associate closely with the pellicle and have an area significantly less than is observed in macrogametocytes (Table I). Compression of nuclear material in microgametocytes has been reported in *H. payevskyi* and *H. micronuclearis* (see Remarks to *H. micronuclearis*). *Haemoproteus nucleofascialis* can be readily distinguished from these parasites by the following characters: (1) the presence of growing gametocytes (of a size greater than erythrocyte nuclei) that do not touch the envelope of erythrocytes along their entire margin (Figs. 17, 18, 25, 26), (2) the terminal position of nuclei in macrogametocytes (Figs. 17, 19, 22), and (3) the band-like shape of microgametocyte nuclei that are located close to the erythrocyte envelope. None of these readily distinguishable features is seen in *H. payevskyi* or *H.*



FIGURES 17–32. *Haemoproteus* (*Parahaemoproteus*) *nucleofascialis* sp. nov. from the blood of red-headed malimbe (*Malimbus rubricollis*). (**17–19**) Young gametocytes. (**20–24**) Macrogametocytes. (**25–32**) Microgametocytes. Long arrows, nuclei of parasites. Short arrows, unfilled spaces among growing gametocytes and envelope of infected erythrocytes. Arrowheads, clumps of volutin. Giemsa-stained thin blood films. Bar = 10 μm.

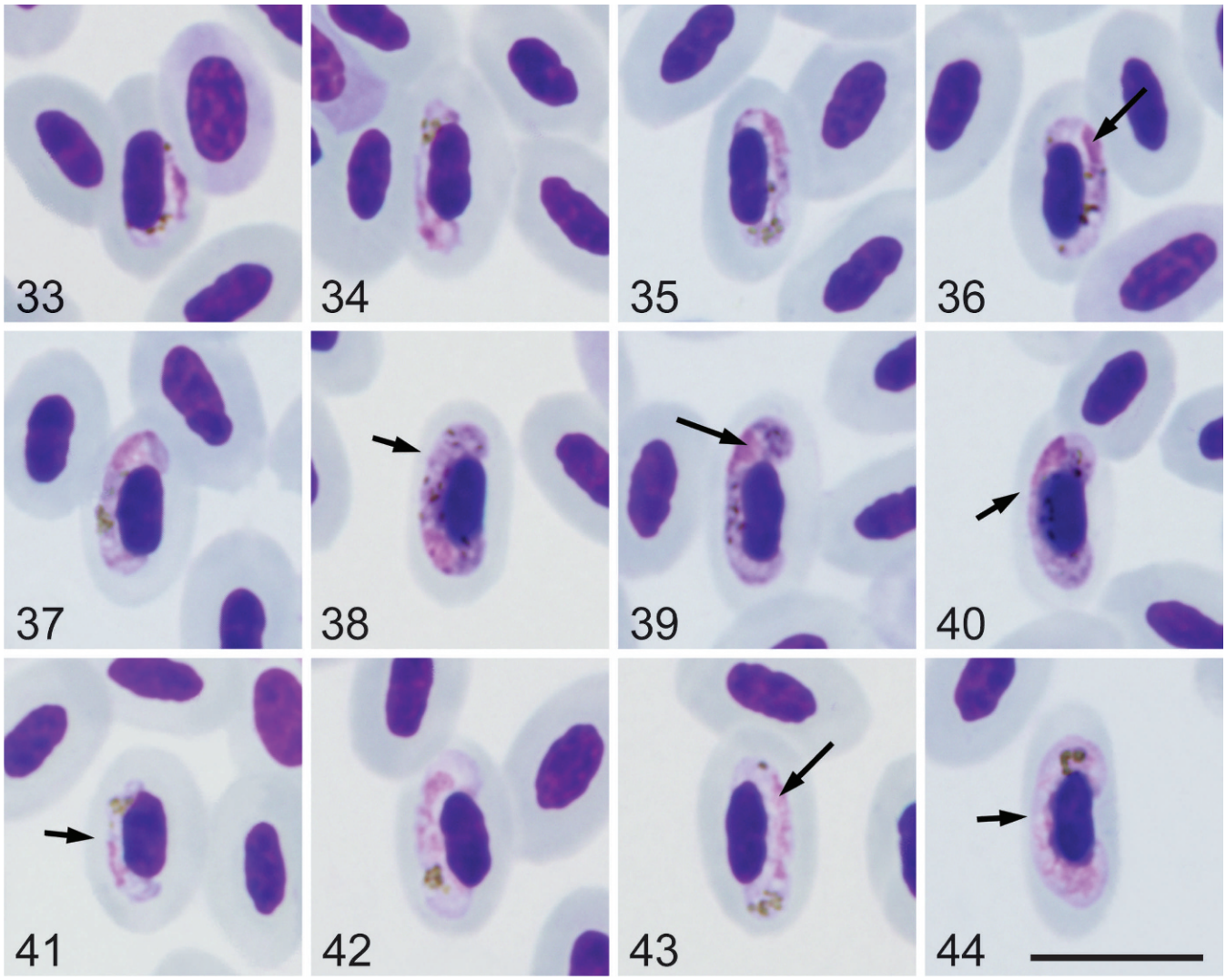
micronuclearis. The genetic distance between different cyt *b* gene lineages of *H. nucleofascialis* and *H. payevskyi* range from 5.1 to 6.0%, and the distance between *H. nucleofascialis* and *H. micronuclearis* lineages range from 2.3 to 4.1%.

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

(Figs. 33–44; Table I)

Diagnosis: Young gametocytes (Fig. 33): Develop in mature erythrocytes, usually lateral to the nuclei of infected erythrocytes, lying slightly asymmetrically to nuclei, so that 1 end of nucleus covered by parasites more than other end (Fig. 33). From early stages of development, gametocytes closely appressed to erythrocyte nuclei but do not touch envelopes of erythrocytes along their entire margin. Pigment granules small (<0.5 μm), golden brown, and tend to group. Volutin granules not observed. Outline of growing gametocytes even or slightly irregular. Influence of gametocytes on infected erythrocytes not pronounced.

Macrogametocytes (Figs. 34–40): Extend around nuclei of erythrocytes, with slender halteridial bodies (<1.5 μm in width in average) and even (Figs. 38–40) or slightly irregular (Figs. 34–36) outlines. Cytoplasm



FIGURES 33–44. *Haemoproteus* (*Parahaemoproteus*) *paranucleophilus* sp. nov. from the blood of red-headed malimbe (*Malimbus rubricollis*). (**33**) Young gametocyte. (**34–40**) Macrogametocytes. (**41–44**) Microgametocytes. Long arrows, nuclei of parasites. Short arrows, unfilled spaces among gametocytes and envelope of infected erythrocytes. Giemsa-stained thin blood films. Bar = 10 μm.

blue, homogeneous in appearance, and lacking volutin granules and visible vacuoles. A few vacuole-like light spaces seen in some gametocytes (Fig. 37). Gametocytes closely appressed to nuclei of erythrocytes but do not touch envelope of erythrocytes along entire margin; slightly displace nuclei of erythrocytes laterally and enclose nuclei up to three-fourths of their circumference. Dumbbell-shaped (with thickenings at ends) and circumnuclear gametocytes absent. Even fully grown gametocytes do not extend to envelope of erythrocytes and do not fill erythrocytes up to their poles. Thus, prominent unfilled space present between gametocytes and envelope of infected erythrocytes (Figs. 38–40). Parasite nucleus prominent (Table I), variable in form, frequently irregular in shape, and subterminal in position (Figs. 36–40). Nucleolus not observed. Pigment granules of medium size (0.5–1.0 μm), roundish or oval, golden brown, and usually randomly scattered throughout cytoplasm (Figs. 36, 38, 39) but sometimes also grouped. Outline of gametocytes even (Figs. 37–40) or slightly irregular (Figs. 34–36), but more frequently the former. Influence of fully grown gametocytes on infected erythrocytes only slightly, if at all, visible (Table I).

Microgametocytes (Figs. 41–44): General configuration as for macrogametocytes with usual hemosporidian sexually dimorphic characters. Nucleus markedly diffuse and therefore difficult to measure. Diffusion of nuclear material increases as parasite matures (compare Figs. 41–44), resulting in rose-colored cytoplasm in advanced gametocytes (Fig. 44). Pigment granules lighter in color than those of macrogametocytes. Gather close to ends of gametocytes and usually grouped (Figs. 41–43) or aggregated in prominent masses (Fig. 44), making them difficult to count. Area of such pigment aggregations varies between 0.9 and 1.7 μm^2 (1.3 ± 0.2 μm^2 on average).

Taxonomic summary

Type host: Red-headed malimbe (*Malimbus rubricollis* L. [Passeriformes, Ploceidae]).

Type locality: Kibale National Park (0°34.7′N, 30°21.3′E, 1,580 m above sea level), Uganda.

Type specimens: Hapantotype (accession 6464 NS, intensity of parasitemia is approximately 0.01%, lineage HV43, GenBank HQ386242, *M. rubricollis*, Kibale National Park, Uganda, collected by G. Valkiūnas, 12 July 2003) is deposited in the Institute of Ecology, Nature Research Centre. Fully grown gametocytes are marked by circles on the hapantotype slide.

DNA sequences: Only 1 lineage has been recorded (see *Type specimens*).

Site of infection: Mature erythrocytes; no other data.

Prevalence: One of 1 investigated red-headed malimbe was infected in the type locality.

Distribution and additional hosts: According to the GenBank data, closely related lineage WAH38 (EU810752, genetic distance between the lineage of *H. paranucleophilus* is 0.02%) was recorded in Vieillot's black weaver and village weaver (*Ploceus cucullatus*) in Gabon (Beadell et al., 2009). It is probable that this parasite is widespread in sub-Saharan Africa.

Etymology: The species name reflects the similarity in morphological features of gametocytes of this parasite to those of *H. nucleophilus*.

Remarks

The most distinctive feature of *H. paranucleophilus* is the presence of a prominent unfilled space between the pellicle of the gametocyte and the envelope of the infected erythrocyte at all stages of development in the blood (Figs. 33–44). This parasite should be distinguished from avian hemoproteid species with halteridial gametocytes that are closely appressed to the erythrocyte nuclei and do not touch the erythrocyte envelope along their entire margin at all stages of their development, including fully grown gametocytes. Three hemoproteid species with such gametocytes parasitize birds, i.e., *H. bilobata*, *H. philippinensis*, and *H. nucleophilus* (see Valkiūnas, 2005). *Haemoproteus bilobata* and *H. philippinensis* can be readily distinguished from *H. paranucleophilus* primarily due to the presence of markedly dumbbell-shaped (with prominent thickenings at the ends) mature gametocytes; such gametocytes are absent from the latter species.

Gametocytes of *H. paranucleophilus* are particularly similar to *H. nucleophilus* (Bennett and Bishop, 1990), which is reflected in the species name. Pigment granules in mature gametocytes of *H. nucleophilus* frequently are gathered in groups of rosette-like, fan-like, star-like, or another form. This is not a character of *H. paranucleophilus*.

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

(Figs. 45–60; Table I)

Diagnosis: Young gametocytes (Figs. 45, 53): Earliest gametocytes indistinguishable from same stages of *H. belopolskyi* and *H. parabelopolskyi*, as described by Valkiūnas (2005) and Valkiūnas et al. (2007), respectively.

Macrogametocytes (Figs. 46–52): Mode of growth, position in infected erythrocytes, and other morphological features of macrogametocytes indistinguishable from same stages of *H. belopolskyi* and *H. parabelopolskyi*, as described by Valkiūnas (2005) and Valkiūnas et al. (2007), respectively, with 1 exception. The average area of macrogametocyte nuclei (Table I) approximately 1.5 times greater ($P < 0.001$) than in *H. parabelopolskyi* (Valkiūnas et al., 2007).

Microgametocytes (Figs. 53–60): General configuration as for macrogametocytes with usual sexual dimorphic characters. Mode of growth and position in infected erythrocytes as well as other morphological features of microgametocytes indistinguishable from same features of *H. belopolskyi* and *H. parabelopolskyi*, as described by Valkiūnas (2005) and Valkiūnas et al. (2007), respectively, with 1 exception. Average number of pigment granules in microgametocytes of *H. homobelopolskyi* (Table I) significantly greater than in microgametocytes of *H. belopolskyi* (the number is 11; $P < 0.001$) and *H. parabelopolskyi* (the number is 9; $P < 0.001$).

Taxonomic summary

Type host: Black-headed weaver (*Ploceus melanocephalus* L. [Passeriformes, Ploceidae]).

Type locality: Queen Elizabeth National Park (0°17.8′S, 30°3.0′E, 1,000 m above sea level) Uganda.

Type specimens: Hapantotype (accession 6937 NS, intensity of parasitemia is 3%, lineage HV42, GenBank HQ386241, *P. melanocephalus*, Queen Elizabeth National Park, Uganda, collected by G. Valkiūnas, 19 July 2003) is deposited in the Institute of Ecology, Nature Research Centre. Parahapantotypes (accessions 6938 NS and G465461) are deposited in the Institute of Ecology, Nature Research Centre, and in the Queensland Museum, respectively. Fully grown gametocytes are marked by circles on the hapantotype and parahapantotype slides.

Additional material: Three blood films (accessions G465462, G465463, and G465464), intensity of parasitemia is 0.2%, *Ploceus pelzelni*, Queen Elizabeth National Park, Uganda, 0°17.8′S, 30°3.0′E, lineage HV41, GenBank accession no. HQ386240, collected by G. Valkiūnas, 19 July 2003, are deposited in the Queensland Museum.

DNA sequences: Mitochondrial cyt *b* lineages HV41 and HV42 (GenBank HQ386240 and HQ38641, respectively).

Site of infection: Mature erythrocytes; no other data.

Prevalence: Two of 2 investigated black-headed weavers were infected in the type locality.

Distribution and additional hosts: According to our study and the GenBank data, lineage HV40 was recorded in 1 slender-billed weaver in the type locality and in 1 Vieillot's weaver in Cameroon. In addition, a closely related lineage WAH34 (EU810732; genetic distance from the lineages of *H. homobelopolskyi* is between 0.010 and 0.013%) was recorded in Vieillot's weaver in Gabon (Beadell et al., 2009). It is probable that this parasite has wide distribution in sub-Saharan Africa. So far, this parasite has been recorded only in species of *Ploceus*.

Etymology: The species name reflects the marked similarity of morphological and morphometric features of gametocytes of this parasite to those of *H. belopolskyi* and *H. parabelopolskyi*.

Remarks

Morphology of *H. homobelopolskyi* n. sp. was compared with the hapantotype specimens of *H. belopolskyi* and *H. parabelopolskyi* (accessions 435.85p and 15317 NS in the collection of Institute of Ecology, Nature Research Centre, respectively). The most distinctive feature of development of *H. homobelopolskyi* is the presence of circumnuclear or close to circumnuclear gametocytes, in which the pellicle does not extend to the erythrocyte envelope; this causes a "dip" and gives the gametocyte a dumbbell-like appearance (Fig. 52). Such dips have been recorded in growing gametocytes of many species of avian hemoproteids, but they are exceptionally rare in circumnuclear forms. Based on these characters, *H. homobelopolskyi* can be readily distinguished from all other avian hemoproteids, except for *H. belopolskyi* and *H. parabelopolskyi*. The main morphological differences among these 3 species are given in the description of *H. homobelopolskyi*. Importantly, fully grown microgametocytes of *H. belopolskyi*, *H. parabelopolskyi*, and *H. homobelopolskyi* never assume the dumbbell-like appearance (Figs. 59, 60); thus, they are different in this character from their macrogametocytes (Figs. 50, 52).

The genetic distance in cyt *b* gene between 2 lineages of *H. homobelopolskyi*, on one hand, and the lineages of *H. belopolskyi* or *H. parabelopolskyi*, on the other hand (see Fig. 61), ranges between 6.0 and 7.7% and 6.3 and 7.1%, respectively. Thus, *H. homobelopolskyi* can be readily distinguished from both *H. belopolskyi* and *H. parabelopolskyi* also based on their cyt *b* sequences.

Phylogenetic relationships of parasites

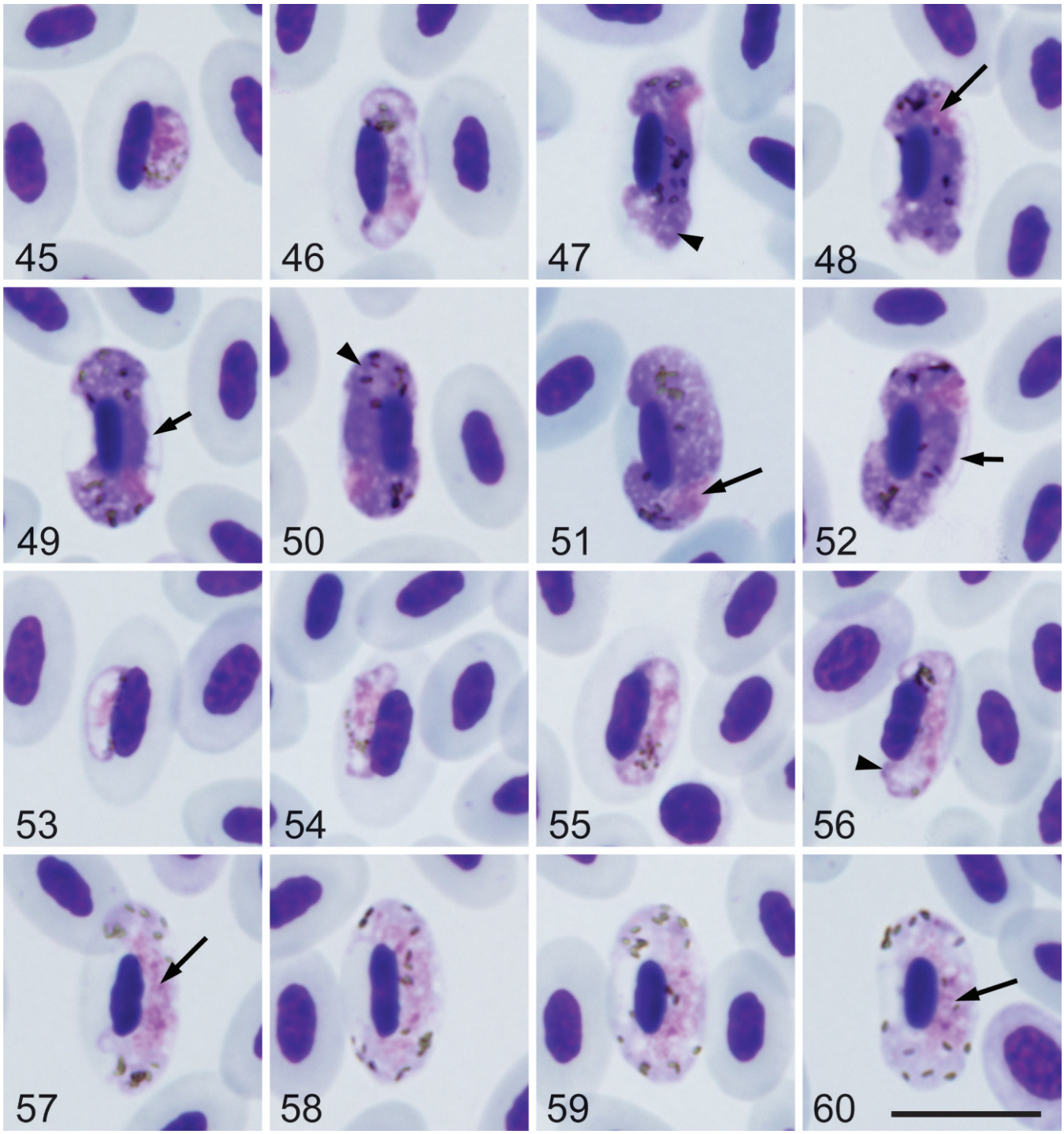
All new species of avian hemoproteids are clearly distinguishable in the phylogenetic tree (Fig. 61, clade B), which corresponds to their morphological differences. In addition, they form a distinct clade and seem to be related phylogenetically based on cyt *b* gene analysis. All lineages of the new species cluster with lineages of *Culicoides* spp.-transmitted species of *Haemoproteus* (*Parahaemoproteus*) spp., so they probably belong to the subgenus *Parahaemoproteus*.

Because parasites of 2 *H. homobelopolskyi* lineages, 5 *H. micronuclearis* lineages, and 2 *H. nucleofascialis* lineages are closely related (Fig. 61) and are indistinguishable based on morphology of their blood stages, we consider these lineages as intraspecies genetic variation of the corresponding morphospecies. Genetic distance in cyt *b* gene among different lineages of *H. micronuclearis* ranges between 0.4 and 1.6%; it is <0.6% for the majority of lineages of this parasite. The genetic distance of this gene between 2 lineages of *H. homobelopolskyi* is 0.6% but is 2.1% between 2 lineages of *H. nucleofascialis*.

The genetic distance among lineages of 4 new species of hemoproteids ranges between 1 and 4.7%. The genetic distance among new species and other readily morphologically distinguishable hemoproteid species, shown in Figure 61, ranges between 2.7 and 14.3% and is >5% for the majority of these species.

DISCUSSION

Haemoproteus micronuclearis, *H. nucleofascialis*, *H. paranucleophilus*, and *H. homobelopolskyi* were attributed to the subgenus *Parahaemoproteus* because cyt *b* lineages of this parasite



FIGURES 45–60. *Haemoproteus* (*Parahaemoproteus*) *homobelopolskyi* sp. nov. from the blood of black-headed weaver (*Ploceus melanocephalus*). (**45, 53**) Young gametocytes. (**46–52**) Macrogametocytes. (**54–60**) Microgametocytes. Long arrows, nuclei of parasites. Short arrows, dips between pellicle of gametocytes and envelope of erythrocytes. Arrowheads, clamps of volutin. Giemsa-stained thin blood films. Bar = 10 μm.

cluster well with the lineages of *Culicoides* spp.-transmitted species of hemoproteids but not to the lineages of the hippoboscid-transmitted species *Haemoproteus columbae* and *Haemoproteus multipigmentatus* that belong to the subgenus *Haemoproteus* (Fig. 61). In addition, only hemoproteids of the subgenus *Parahaemoproteus* spp. are known to develop in passeriform birds (Valkiūnas, 2005). Hemoproteids of the subgenera *Parahaemoproteus* and *Haemoproteus* are transmitted by species of Ceratopogonidae and Hippoboscidae, respectively. They undergo different modes of gametogenesis and sporogony in the vectors (Bennett et al., 1965; Atkinson, 1991; Valkiūnas, 2005); thus, they usually present in different clades in phylogenetic trees based on cyt *b* gene (Martinsen et al., 2008; Iezhova et al., 2010; Santiago-Alarcon et al., 2010; Valkiūnas, Santiago-Alarcon et al., 2010).

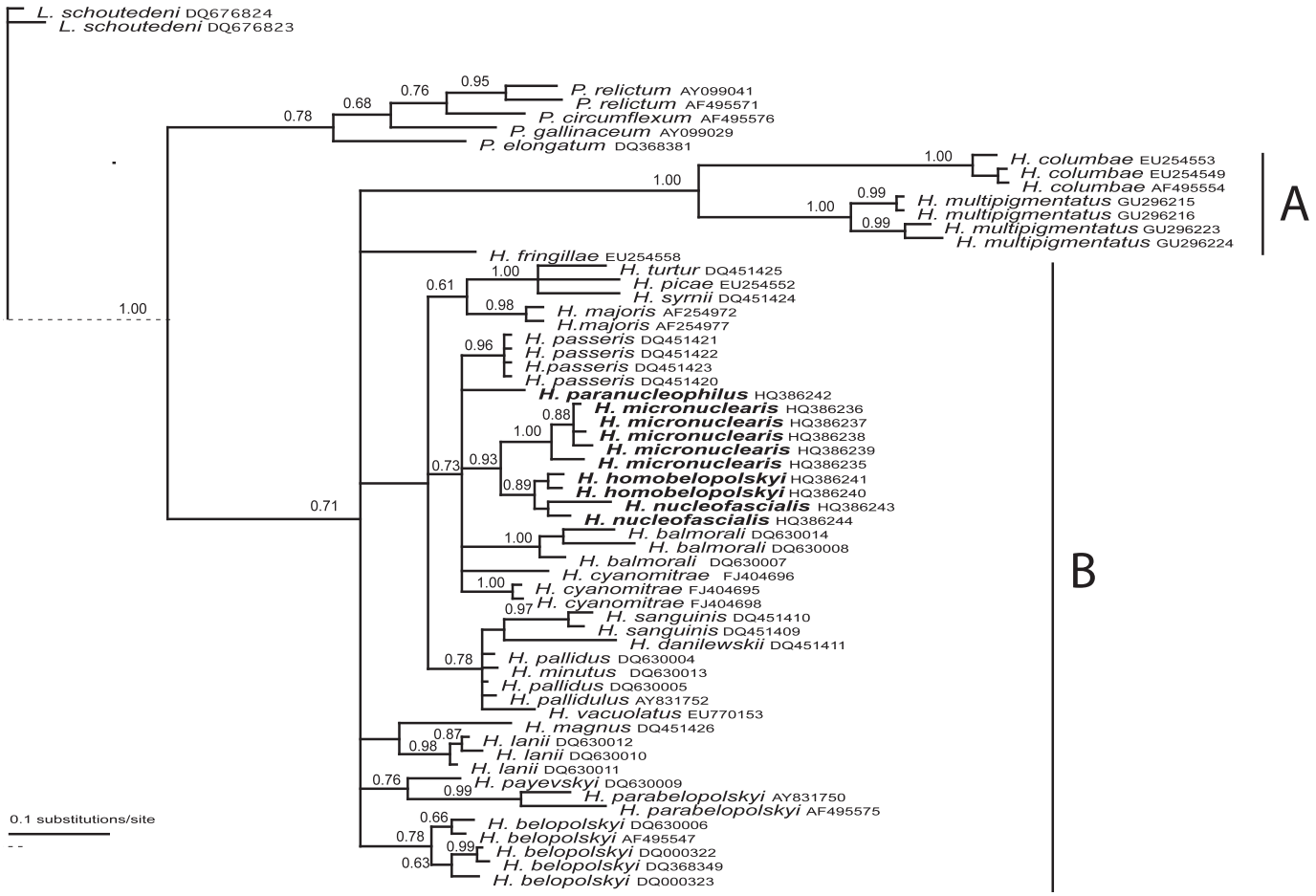


FIGURE 61. Bayesian phylogeny of 53 mitochondrial cytochrome *b* lineages of *Haemoproteus* spp. and 5 lineages of *Plasmodium* spp. Two lineages of *Leucocytozoon schoutedeni* are used as outgroups. Posterior probabilities >0.6 are indicated near the nodes. GenBank accessions are given after parasite species names, with the names of new species in bold. Branch lengths are drawn proportionally to the amount of changes (scale bars are shown). Vertical bars A and B indicate hemoproteid species belonging to the subgenera *Haemoproteus* and *Parahaemoproteus,* respectively.

According to most recent phylogenies (Santiago-Alarcon et al., 2010; Valkiūnas, Santiago-Alarcon et al., 2010), *Parahaemoproteus* and *Haemoproteus* are sister groups of avian hemoproteids, so the traditional subgeneric classification of avian hemoproteids (see Levine and Campbell, 1971; Valkiūnas, 2005) remains valid. Vector species for *H. micronuclearis*, *H. nucleofascialis*, *H. paranucleophilus*, and *H. homobelopolskyi* need to be identified; phylogenetic relationships of detected lineages (Fig. 61) show that *Culicoides* spp. should be incriminated first.

We used only positively identified morphospecies of avian hemoproteids in the phylogenetic analysis (Fig. 61). Genetic distances among all cyt *b* lineages of 4 new species, on the one hand, and the lineages of hippoboscid-transmitted *H. columbae* and *H. multipigmentatus*, on the other hand (Fig. 61, clade A), are >11%. Genetic divergence among the lineages of 4 new species, on the one hand, and the lineages of other morphospecies belonging to the subgenus *Parahaemoproteus*, on the other hand (Fig. 61, clade B), is >5% for the majority of the readily distinguishable morphospecies. This is in accordance with the hypothesis of Hellgren et al. (2007) and recent data from Iezhova et al. (2010) and Valkiūnas, Sehgal et al. (2010) that hemosporidian species with a genetic distance greater than 5% in the mitochondrial cyt *b* gene tend to be morphologically differentiated. However, this pattern works only 1 direction; there are numerous readily distinguishable morphospecies with genetic divergence <5% among their lineages, and as small as <1% in some species (see Hellgren et al., 2007; Valkiūnas et al., 2009; Iezhova et al., 2010), as also was recorded during this study. Interestingly, even in cases of great genetic distance in the cyt *b* gene, morphological differentiation might be small and visible in single characters but still readily distinguishable, as is the case among *H. homobeloposkyi*, *H. belopolskyi*, and *H. parabelopolskyi* (see Valkiūnas et al., 2007). Accumulation of additional data will improve the understanding of phylogenetic trees based on the cyt *b* gene, which is shown to be informative in numerous taxonomic, ecological, and evolutionary biology studies of avian hemosporidians (Perkins and Schall, 2002; Križanauskienė et al., 2006; Sehgal et al., 2006; Palinauskas et al., 2007; Beadell et al., 2009; Bensch et al., 2009; Svensson and Ricklefs, 2009; Iezhova et al., 2010; Santiago-Alarcon et al., 2010).

Bennett et al. (1972, 1991) proposed a convenient taxonomic device that morphologically similar hemoproteids should be described and named as new species if they were found in birds of different families or even subfamilies. This device was used successfully in the beginning of taxonomic revision of the Haemoproteidae and also the Leucocytozoidae (Valkiūnas and

Ashford, 2002); it is still in use in the development of *Haemoproteus* taxonomy at the species level (Barraclough et al., 2008; Parsons et al., 2010). However, both molecular investigations (Fallon et al., 2003, 2005; Szymanski and Lovette, 2005; Beadell et al., 2009; Latta and Ricklefs, 2010) and molecular studies combined with microscopy data (Križanauskienė et al., 2006; Svensson and Ricklefs, 2009; Križanauskienė et al., 2010) show that many *Haemoproteus* spp. lineages are present and produce fully grown gametocytes in birds belonging to different families of Passeriformes. *Haemoproteus majoris* and *H. coatneyi* are the best investigated hemoproteids inhabiting passeriform birds belonging to several phylogenetically distant families in the Palearctic and Nearctic, respectively (Križanauskienė et al., 2006; Svensson and Ricklefs, 2009). Importantly, data from these field studies are in accord with former experimental research that showed successful development to gametocyte stage of *Haemoproteus mansoni* (=*Haemoproteus meleagridis*) in birds belonging to different families/subfamilies of galliform birds (Atkinson, 1986) and *Haemoproteus fringillae* in passeriform birds belonging to the Fringillidae and Emberizidae (Valkiūnas, 2005).

It should be noted that numerous *Haemoproteus* spp. lineages have been recorded only in closely related birds of the same family (Križanauskienė et al., 2006; Beadell et al., 2009). Presumably, hemoproteids in hosts of different avian families might be different species or subspecies. However, that certainly is not a rule (see Svensson and Ricklefs, 2009; Križanauskienė et al., 2010; Latta and Ricklefs, 2010); so, to be accepted as valid species, hemosporidians also should be shown to be different in characters other than natural host range.

It is worth noting that the narrow host range of avian hemoproteids recorded in some field studies might be illusory, in part due to the following reasons (Križanauskienė, 2010). First, the relatively high feeding specialization of *Parahaemoproteus* spp. vectors (biting midges of the *Culicoides*). Many biting midge species are more specialized to take bloodmeals on particular birds than some mosquito species (Glukhova, 1989; Atkinson, 1991; Valkiūnas, 2005). For example, subspecies of *Culex pipiens* group are widely distributed host generalists and are excellent vectors of many species of avian malaria (Kim and Tsuda, 2010; Kimura et al., 2010). Thus, the feeding behavior of biting midges may be subject to ecological constraints in some environments that promote feeding on many species of birds. Ecosystems that are particularly rich with respect to vector species and avian hosts (e.g., tropical ecosystems) might favor greater diversity in lineages of avian hemoproteids that are found in a few avian hosts. This warrants further investigation. Second, there is great variation in sampling intensity of particular hosts in different studies, especially the studies that pool host samples across broad geographic regions, often across continents. This may bias information about host distribution of lineages, particularly in relation to the first statement. In addition, the number of lineages from a host species depends on sample size; it increases with the total number of hosts investigated or infected (Latta and Ricklefs, 2010). Transmission of the same lineages of some *Haemoproteus* species among birds belonging to different families can easily be detected during population studies on a small geographic scale, particularly in relatively simple island ecosystems that are easy to control (see Fallon et al., 2003, 2005; Szymanski and Lovette, 2005; Križanauskienė et al., 2006; Svensson and Ricklefs, 2009; Križanauskienė et al., 2010; Latta and Ricklefs, 2010). It seems that host shifts of *Haemoproteus* spp. between birds of different families are more common in wildlife (Križanauskienė, 2010) than previously suggested (Bennett et al., 1972, 1991).

Despite the limited information on vertebrate host specificity for the majority of species of avian hemoproteids and other hemosporidians, it is now clear that the level of specificity markedly varies among different *Haemoproteus* species in birds, as is the case with other avian hemosporidian parasites, including *Plasmodium* spp. (Valkiūnas, 2005; Palinauskas et al., 2008; Beadell et al., 2009; Valkiūnas et al., 2009). There are strictly specific hemoproteid species (e.g., *H. minutus* and *H. payevskyi* that parasitize mainly the blackbird (*Turdus merula*) and several species of *Acrocephalus*, respectively) and parasites of broad specificity (e.g., *H. majoris* parasitizing passeriform birds belonging to the Sylviidae, Paridae, Fringillidae, and Muscicapidae; see Hellgren et al., 2007; Bensch et al., 2009; Križanauskienė et al., 2010). Furthermore, even if certain hemosporidian lineages are reported only in birds of 1 family, they usually do not infect representatives of all genera but instead are restricted to certain groups of birds (see MalAvi database, Bensch et al., 2009). More than 50 *Haemoproteus* species were described primarily using the host-family specificity device in birds of the Passeriformes alone (Valkiūnas, 2005). However, due to recent numerous records of the same *Haemoproteus* spp. lineages in birds of different families, it is clear that natural host range can hardly be accepted as a reliable taxonomic character alone. The validity of many hemosporidian species that were described using the host-family specificity device should be confirmed by other characters. This is particularly evident in parasites of passeriform birds, but it also was documented experimentally with *H. mansoni* that infects a wide range of galliform birds (Atkinson, 1986). Thus, the naming of hemoproteid species based primarily on records of morphologically similar parasites in birds of different families is likely putative and provisional. It should be questioned and, preferably, discontinued.

During the description of new hemoproteid species, it is important to compare the morphology of the parasites to that of already known species in the corresponding subgenus. This is a long existing practice in the study of avian *Plasmodium* species. Comparison of parasites developing in birds of the same order is particularly important because there is strong molecular and experimental evidence that transmission of the same lineages of avian *Haemoproteus* spp. occurs among birds of different families but of the same order (Fallon et al., 2005; Szymanski and Lovette, 2005; Valkiūnas, 2005; Križanauskienė et al., 2006, 2010; Svensson and Ricklefs, 2009). We did such a comparison during the description of new species in this study in spite of the fact that so far all new species have been reported in birds of the Ploceidae. Such taxonomic work requires the use of a wide range of morphological characters of parasites, good-quality morphological material, and standardization of morphological descriptions and molecular markers.

The main obstacle in the identification of hemosporidian species is light parasitemia in naturally infected birds (Valkiūnas, 2005). That is why bar-coding of hemosporidian species remains an important task, particularly in wildlife malaria studies (Bensch et al., 2009). Optimistically, recent advances in PCR-based techniques, which are already routine procedures accessible in the majority of research laboratories, provide opportunities to determine lineages of parasites. Then, using the lineage informa-

tion, it is possible to link light parasitemias of the same parasite in different samples with the aim of gaining information about the full range of blood stages for morphological analysis. Moreover, new molecular techniques provide opportunities to describe taxonomic characters in the parasite genomes on the level of single cells (Palinauskas et al., 2010), offering an exciting opportunity to develop taxonomy of blood parasites, particularly during natural co-infections.

We encourage the deposition of all good-quality voucher specimens of hemosporidian parasites in well-recognized museums, as well as provision of the accession numbers of in press specimens and ideally also in GenBank. This would make identifications of hemosporidian species easily repeatable and would save material for future taxonomic and other studies. Such samples are time-consuming and expensive to obtain due to the difficulties of fieldwork, particularly in tropical countries.

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