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TWO NEW *HAEMOPROTEUS* SPECIES (HAEMOSPORIDA: HAEMOPROTEIDAE) FROM COLUMBIFORM BIRDS

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ABSTRACT: Here we describe *Haemoproteus (Haemoproteus) multivolutinus* n. sp. from a tambourine dove (*Turtur timpanistria*) of Uganda and *Haemoproteus (Haemoproteus) paramultipigmentatus* n. sp. (Haemosporida, Haemoproteidae) from the Socorro common ground dove (*Columbina passerina socorroensis*) of Socorro Island, Mexico. These parasites are described based on the morphology of their blood stages and segments of the mitochondrial cytochrome *b* gene that can be used for molecular identification and diagnosis of these species. Gametocytes of *H. multivolutinus* possess rod-like pigment granules and are evenly packed with volutin, which masks pigment granules and darkly stains both macro- and microgametocytes in the early stages of their development. Based on these 2 characters, *H. multivolutinus* can be readily distinguished from other species of hemoproteids parasitizing columbiform (Columbiformes) birds. *Haemoproteus paramultipigmentatus* resembles *Haemoproteus multipigmentatus*; it can be distinguished from the latter parasite primarily due to the broadly ovoid shape of its young gametocytes and significantly fewer pigment granules in its fully developed gametocytes. We provide illustrations of blood stages of the new species, and phylogenetic analyses identify DNA lineages closely related to these parasites. Cytochrome *b* lineages of *Haemoproteus multivolutinus* and *H. paramultipigmentatus* cluster with hippoboscid-transmitted lineages of hemoproteids; thus these parasites likely belong to the subgenus *Haemoproteus*. We emphasize the importance of using cytochrome *b* sequences in conjunction with thorough microscopic descriptions to facilitate future identification of these and other avian hemosporidian species.

The hemosporidian parasites of the genus *Haemoproteus* from birds make up 2 subgenera, *Haemoproteus* and *Parahaemoproteus*. The vast majority of species of the genus belong to the *Parahaemoproteus*, found in many songbirds and most other orders of birds (Valkiūnas, 2005). Species of the subgenus *Haemoproteus* are typically found in columbiform birds (Columbiformes) and have been recently found in seabirds (Levin et al., 2012; Merino et al., 2012). There are presently only 6 described species in the subgenus *Haemoproteus* that infect pigeons and doves, but it is likely that more undescribed species exist. With recent trends in combining microscopy with molecular sequencing, taxonomic classification of hemosporidian parasites has enjoyed a renaissance, resulting in many new comprehensive descriptions. For examples of parasite lineage linkage to their morphospecies and additional literature on this subject, see Martinsen et al. (2006), Hellgren et al. (2007), Valkiūnas et al. (2007), Valkiūnas et al. (2010), Iezhova et al. (2011), Levin et al. (2012), and Merino et al. (2012). Here we add 2 more *Haemoproteus* species to the list of parasites found in doves.

During studies on the distribution and evolutionary biology of pathogens in Uganda (Valkiūnas et al., 2005) and of Socorro Island, Mexico (Carlson et al., 2011; Yanga et al., 2011), blood samples were collected from the tambourine dove *Turtur timpanistria* and the Socorro common ground dove *Columbina passerina socorroensis*, respectively. Two previously undescribed species of *Haemoproteus* (Haemosporida, Haemoproteidae) were found during these studies. These parasites are named and described here using data on the morphology of their blood stages, and partial sequences of the mitochondrial cytochrome *b* (cyt *b*) gene.

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MATERIALS AND METHODS

Collection of blood samples

Blood samples were collected in Uganda and from Socorro Island. In Uganda, we sampled 1 tambourine dove during the dry season in 2003 (for sampling details see Valkiūnas et al., 2005). We caught 23 Socorro common ground doves on Socorro Island (for sampling details see Carlson et al., 2011; Yanga et al., 2011). All birds were caught with mist nets; they were ringed, bled, and released. The blood was taken by puncturing the brachial vein. Approximately 50 µl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were preserved 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. 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. 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

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution, 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). The morphology of new species was compared with the type and voucher specimens of hemoproteids of the subgenus *Haemoproteus* from their type vertebrate hosts belonging to the Columbidae: *Haemoproteus columbae* (host is the rock dove *Columba livia*, nos. 2905.87, 47723 NS, 47724 NS in Collection of Institute of Ecology, Nature Research Centre, hereafter CNRC), *Haemoproteus multipigmentatus* (Galapagos dove *Zenaida galapagoensis*, accession nos. 47725 NS, 47726 NS in the CNRC), *Haemoproteus palumbis* (woodpigeon *Columba palumbus*, 969, 970 in the Natural History Museum, London, U.K. and no. 2067.87 in the CNRC), *Haemoproteus sacharovi* (mourning dove *Zenaida macroura*, nos. 45236A, 45236B, 103700 in Queensland Museum, Queensland, Australia, and no. 47739 in the CNRC), and *Haemoproteus turtur* (turtle dove *Streptopelia turtur,* no. 1315.87 in the CNRC). Student's *t*-test for independent samples was used to determine statistical

TABLE I. Morphometry of host cells and mature gametocytes of 2 new species of *Haemoproteus* from columbiform birds.

Feature	Measurements*	
	H. paramultipigmentatus	*H. multivolutinus*
Uninfected erythrocyte		
Length	11.1–12.9 (12.0 ± 0.6)	10.8–13.5 (12.3 ± 0.7)
Width	5.8–7.4 (6.8 ± 0.4)	5.7–7.1 (6.5 ± 0.4)
Area	55.3–75.0 (66.3 ± 5.6)	55.2–72.3 (64.6 ± 5.2)
Uninfected erythrocyte nucleus		
Length	4.8–6.0 (5.4 ± 0.4)	3.8–6.2 (5.0 ± 0.6)
Width	1.9–2.6 (2.2 ± 0.2)	2.1–2.9 (2.4 ± 0.2)
Area	9.0–12.5 (10.6 ± 1.1)	7.9–13.0 (10.0 ± 1.3)
Macrogametocyte		
Infected erythtocyte		
Length	11.6–13.6 (12.7 ± 0.6)	11.5–14.6 (12.9 ± 0.8)
Width	5.3–6.7 (6.0 ± 0.4)	4.9–6.7 (6.2 ± 0.4)
Area	53.8–69.6 (63.3 ± 4.9)	54.3–71.5 (62.8 ± 4.9)
Infected erythrocyte nucleus		
Length	4.1–5.8 (5.0 ± 0.4)	3.7–4.8 (4.3 ± 0.3)
Width	1.9–3.0 (2.3 ± 0.3)	2.0–2.9 (2.4 ± 0.2)
Area	7.9–10.6 (9.6 ± 0.7)	7.8–10.0 (8.7 ± 0.6)
Gametocyte		
Length	12.7–18.6 (15.2 ± 1.7)	13.7–21.0 (18.1 ± 2.1)
Width	2.0–3.3 (2.7 ± 0.3)	2.1–3.1 (2.6 ± 0.3)
Area	32.7–44.5 (36.8 ± 2.9)	34.4–45.7 (40.8 ± 3.0)
Gametocyte nucleus		
Length	1.8–2.8 (2.3 ± 0.3)	1.7–2.8 (2.1 ± 0.3)
Width	1.2–2.3 (1.6 ± 0.3)	1.1–2.2 (1.5 ± 0.3)
Area	2.0–4.2 (3.0 ± 0.7)	1.5–3.8 (2.6 ± 0.6)
Pigment granules	24.0–32.0 (29.0 ± 2.4)	19.0–28.0 (22.9 ± 2.6)
NDR†	0.4–0.8 (0.6 ± 0.1)	0.3–0.9 (0.5 ± 0.2)
Microgametocyte		
Infected erythrocyte		
Length	11.0–14.0 (12.7 ± 0.7)	11.2–13.7 (12.6 ± 0.6)
Width	5.3–7.1 (6.5 ± 0.4)	5.3–6.7 (6.3 ± 0.4)
Area	50.4–80.8 (67.8 ± 6.7)	45.8–69.6 (63.4 ± 5.8)
Infected erythrocyte nucleus		
Length	4.5–6.1 (5.4 ± 0.4)	3.8–5.9 (4.8 ± 0.5)
Width	1.9–2.6 (2.1 ± 0.2)	2.1–2.9 (2.4 ± 0.2)
Area	7.9–11.7 (10.0 ± 1.0)	8.0–12.0 (9.6 ± 0.9)
Gametocyte		
Length	11.8–13.7 (12.7 ± 0.6)	11.1–14.3 (12.4 ± 0.8)
Width	2.3–3.6 (2.8 ± 0.4)	1.9–3.3 (2.6 ± 0.3)
Area	30.5–42.3 (36.8 ± 3.6)	26.9–39.7 (32.7 ± 4.0)
Gametocyte nucleus		
Length	3.8–6.2 (5.0 ± 0.6)	3.1–5.3 (4.2 ± 0.6)
Width	2.1–3.6 (2.8 ± 0.4)	1.8–3.3 (2.6 ± 0.3)
Area	8.7–15.6 (11.7 ± 1.7)	7.5–13.4 (9.9 ± 1.4)
Pigment granules	14.0–22.0 (18.3 ± 2.2)	15.0–22.0 (18.4 ± 2.0)
NDR†	0.4–0.8 (0.6 ± 0.1)	0.3–0.8 (0.6 ± 0.2)

* 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).

significance between mean linear parameters. A *P*-value of 0.05 or less was considered significant.

DNA extraction, 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). 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 5xGoTaq 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 above, but for 35 cycles instead of 20. All reactions were performed in 25 µl volumes and were accompanied by negative (ddH_2O) and positive controls (samples from infected birds, confirmed by microscopy) in order 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). We used the BLAST algorithm to compare the sequences of new lineages to known *Haemoproteus* spp. lineages deposited in GenBank.

Visualization of "double bases" in electropherograms of cyt *b* sequences was used to estimate presence of possible haemosporidian co-infections.

Phylogenetic analysis

We used 21 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 targeted taxonomic and molecular characterization studies.

The phylogenetic tree was created using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The appropriate model of sequence evolution was determined by MrModeltest version 2.3 (Posada and Buckley, 2004) using AIC scores. MrModeltest selected the GTR+I+G model (–lnL 1425.1550), which was used for subsequent Bayesian analysis. Two lineages of avian *Plasmodium* (*P. juxtanucleare* and *P. multivacuolaris*) were selected as outgroups. For the Bayesian analysis we ran 10,000,000 generations using the Markov chain Monte Carlo (MCMC) algorithm. Analyses were done twice: once with aligned sequences of varying lengths, and once with all sequences aligned and grouped into a consensus of 288 bp in length. Sequence alignment was done using Sequencher 4.9 (GeneCodes). Additionally, we performed a maximum likelihood (ML) analysis of the data using PAUP 4.0a112 (Swofford, 2003). The model for ML was generated using MrModeltest 2.3. With both methods of analysis, we obtained phylogenetic trees with identical topologies. The sequence divergence among the lineages was calculated using Kimura 2-parameter settings (Kimura, 1980). The divergence data were generated with PAUP 4.0.a112 (Swofford, 2003).

RESULTS

New species of *Haemoproteus* were found in 1 individual tambourine dove from Uganda, and 10 Socorro common ground doves from Socorro Island, Mexico. We did not detect co-infections in the type material of new species, either by

microscopic examination or by PCR-based detection: thus, descriptions of new species are based on single infections.

DESCRIPTION

Haemoproteus (Haemoproteus) multivolutinus n. sp.

(Figs. 1–16; Table I)

Young gametocytes (Fig. 1): Develop in mature erythrocytes. Earliest forms can be seen anywhere in infected erythrocytes, but more frequently recorded in a sub-polar position relative to erythrocyte nuclei; markedly variable in shape. With development, gametocytes extend laterally along nuclei of erythrocyte, touching neither nuclei nor envelope of erythrocytes. One or several clear small vacuoles present (Fig. 1). Nucleus prominent (Fig. 1). Pigment granules few, small (<0.5 μm), black, and frequently grouped. A clear roundish spot of dark-violet volutin present in early gametocytes (Fig. 1), a characteristic feature of this species development. Amount of volutin increases markedly as gametocytes grow resulting in the presence of several irregularly shaped large groupings of volutin in advanced forms. Outline of growing gametocytes wavy (Fig. 1) or irregular. Influence of young gametocytes on infected erythrocytes usually not pronounced.

Macrogametocytes (Figs. 2–8): Extend along nuclei of erythrocytes; elongate slender bodies with wavy (Fig. 2), irregular or slightly amoeboid (Figs. 4–6) outline. Finger-like outgrowths frequently present at the ends of growing gametocytes (Fig. 3), a characteristic feature of this species development. Numerous large irregular-shape groupings of dark-violet volutin present (Figs. 5, 6), giving dark staining to gametocytes and markedly masking both the cytoplasm and pigment granules; volutin overfills more or less evenly the entire gametocyte (Figs. 2–8), a characteristic feature of this species' development. Small (<0.5 μm) vacuoles frequently seen (Figs. 5, 6). Growing gametocytes, with length exceeding the length of erythrocyte nuclei (Figs. 2, 3), have no permanent position in relation to nuclei or envelope of erythrocytes; usually lying free in cytoplasm, not touching either nuclei or envelope of erythrocytes (Fig. 2); also seen touching nucleus or envelope of erythrocytes (Fig. 3), but usually not both these cellular structures at this stage of development. Advanced gametocytes only slightly displace nuclei of erythrocytes; usually touching both erythrocyte nuclei and envelope, filling erythrocytes up to their poles (Figs. 5–7). Mature gametocytes extend around nuclei of erythrocytes, enclosing them with their ends, but do not encircle nuclei completely (Figs. 6, 7); they usually push nuclei with their middle part to the envelope of erythrocytes and finally occupy nearly the entire cytoplasmic space in host cells (Fig. 8). In advanced gametocytes, 2 clear unfilled spaces appear between the ends of gametocytes and nuclei of erythrocytes (Fig. 7), giving gametocytes a horn-like appearance, and disappearing as the parasite matures (Fig. 8). Fully grown gametocytes closely associated with nuclei and envelope of erythrocytes, filling erythrocytes up to their poles (Fig. 8). Parasite nucleus small (Table I), variable in form, frequently irregular in shape, median or submedian in position (Figs. 7, 8). Nucleolus not seen. Pigment granules of medium size (0.5–1 μm), rod-like or oval, black, numerous (Table I), randomly scattered throughout the cytoplasm. Mature gametocytes are halteridial, they displace nuclei of erythrocytes laterally, occasionally close to envelope of erythrocytes (Fig. 8).

Microgametocytes (Figs. 9–16): General configuration as for macrogametocytes with usual hemosporidian sexually dimorphic characters. Staining is dark and similar to macrogametocytes due to presence of prominent volutin. Pigment granules usually scattered (Figs. 13, 14), but also can be grouped close to ends of gametocytes (Fig. 11), and even aggregated in solid masses (Fig. 16). Number of pigment granules is less than in macrogametocytes (Table I, $P < 0.05$).

Taxonomic summary

Type host: Tambourine dove *Turtur timpanistria* L. (Columbiformes, Columbidae).

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

Type specimens: Hapantotype (accession number 48661 NS, intensity of parasitemia is approximately 0.04%, lineage HV46, GenBank accession no. JX275888, *T. timpanistria*, Kibale National Park, collected by G. Valkiūnas, 14 July 2003) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accession nos. 48662 NS, and USNPC 106051.00, other data as for the hapantotype) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, and in the U.S. National Parasite Collection, Beltsville, Maryland, respectively.

DNA sequences: Mitochondrial cyt *b* lineage HV46 from bird ID no. 23–113 from type material (389 base pairs; GenBank JX275888).

Site of infection: Mature erythrocytes; no other data.

Prevalence: Overall prevalence was 1 of 1 (100%) in Uganda. One of 1 sampled tambourine doves was infected at the type locality.

Distribution and additional hosts: No sequences in GenBank were found to be identical to this lineage.

Etymology: The species name reflects overfilling of both macro- and microgametocytes of this parasite with volutin.

Remarks

Eight species of hemoproteids have been described in the subgenus *Haemoproteus* (Bennett and Peirce, 1990; Valkiūnas, 2005; Valkiūnas et al., 2010; Levin et al., 2012). *Haemoproteus multivolutinus* can be readily distinguished from all these parasites, primarily due to numerous rod-like pigment granules and prominent volutin, which entirely overfills more or less evenly both macro- and microgametocytes (Figs. 2–8, 10–16).

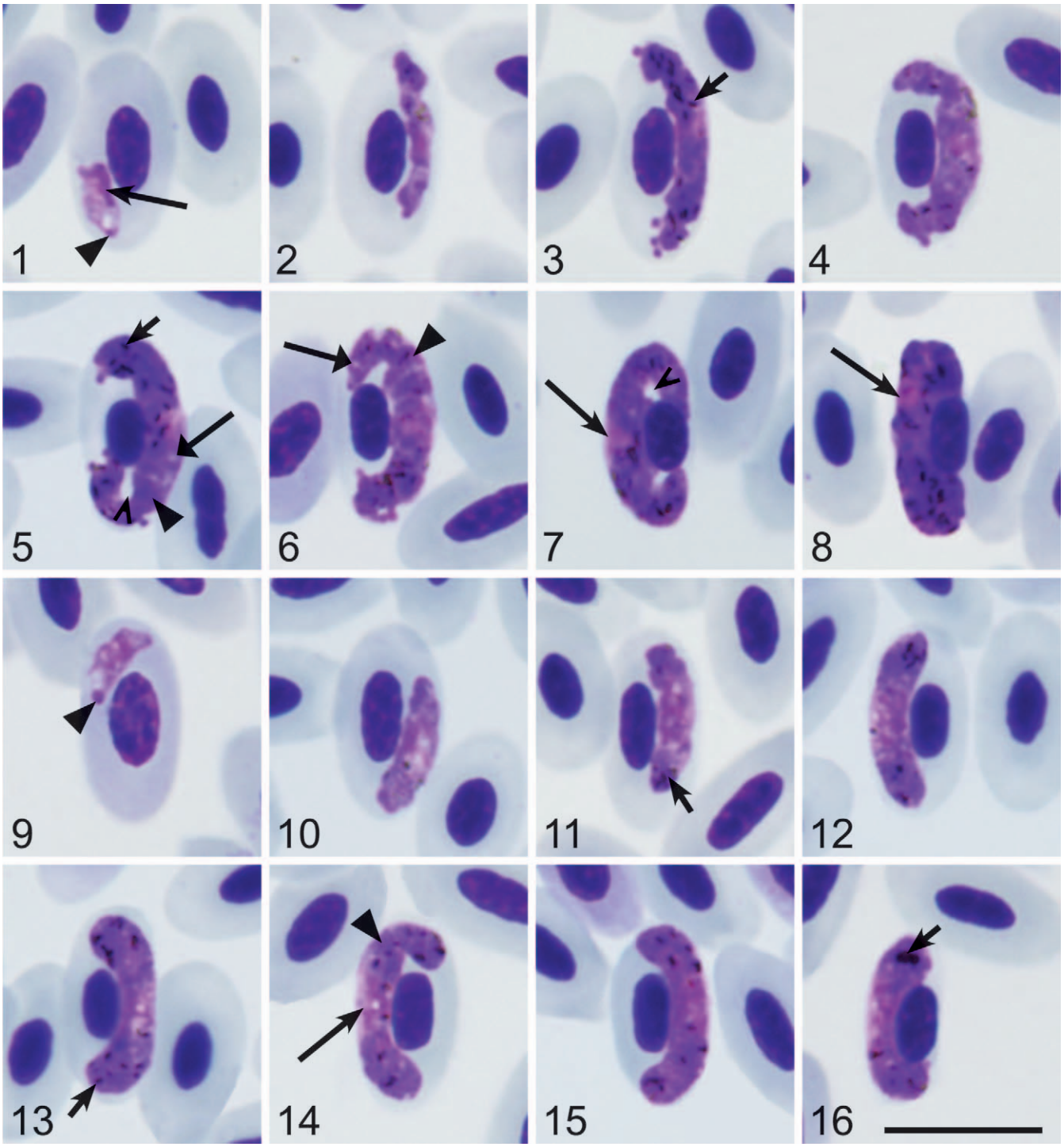
Six species of hemoproteids parasitize doves and pigeons (Figs. 17–47): *H. columbae* (Kruse, 1890), *H. multipigmentatus* (Valkiūnas et al., 2010), *H. palumbis* (Baker, 1966), *H. sacharovi* (Novy and MacNeal, 1904), *H. turtur* (Covaleda Ortega and Gállego Berenguer, 1950), and *H. paramultipigmentatus* n. sp. (see description below). In addition to the rod-like shape of pigment granules, *H. multivolutinus* can be readily distinguished from these parasites due to the following features. In gametocytes of *H. columbae,* volutin and pigment granules tend to aggregate into large round compact masses (Figs. 17–19), which frequently exceed 1 μm in diameter in microgametocytes (Fig. 19). Mature gametocytes of *H. sacharovi* are highly pleomorphic and are outwardly similar to gametocytes of *Leucocytozoon* spp., but they possess fine pigment granules (Figs. 20–22); average width of fully grown gametocytes of this parasite is >5 μm (Valkiūnas, 2005). Mature gametocytes of *H. multipigmentatus* possess numerous (more than 40 on average) pigment granules (Figs. 23–25). Gametocytes of *H. paramultipigmentatus* do not possess visible volutin (see Figs. 32–47). None of these features is characteristic of *H. multivolutinus,* which is particularly similar to *H. palumbis* (Figs. 26–28) and *H. turtur* (Figs. 29–31), so should be compared with these 2 parasites. Prominent volutin is present in gametocytes of these 3 species, but it is gathered mainly on the gametocyte ends in *H. turtur* and *H. palumbis*, but is overdispersed in gametocytes of the new species, which is particularly evident in microgametocytes (compare Figs. 8, 16 with Figs. 26–31).

Haemoproteus (Haemoproteus) paramultipigmentatus n. sp.

(Figs. 32–47; Table I)

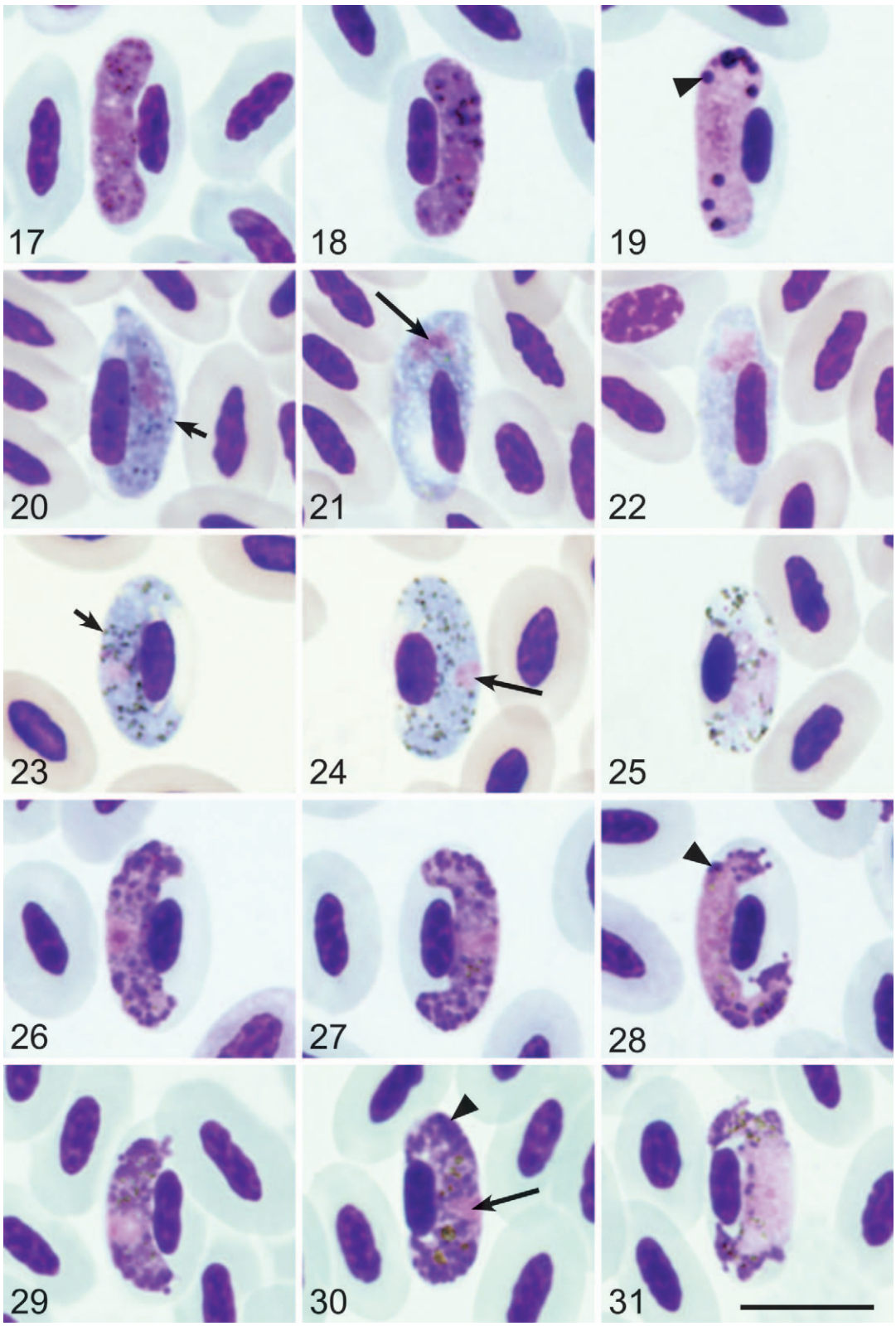
Young gametocytes (Figs. 32, 33): Develop in mature erythrocytes. Earliest forms seen anywhere in infected erythrocytes, but more frequently recorded subpolar to erythrocyte nuclei; broadly ovoid forms predominant (Fig. 32). With development, gametocytes extend along the nuclei of erythrocytes (Fig. 33); they have no permanent position neither to the nuclei nor to the envelope of erythrocytes. The cytoplasm homogenous, lacking visible vacuoles and volutin granules. Nucleus small, of irregular shape. Pigment granules small (<0.5 μm), black, numerous, and usually scattered (Figs. 32, 33). Outline of growing gametocytes wavy (Fig. 32), irregular (Fig. 33), or slightly ameboid. Influence of young gametocytes on infected erythrocytes usually not pronounced.

Macrogametocytes (Figs. 34–42): Extend along nuclei of erythrocytes; elongate slender bodies with wavy, irregular, or slightly ameboid outline. Cytoplasm blue, homogeneous in appearance, occasionally possesses few small vacuoles. Volutin not seen. Growing gametocytes, with length exceeding length of erythrocyte nuclei (Figs. 34–37), have no permanent position in relation to nuclei or envelope of erythrocytes; sometimes lying free in cytoplasm, not touching either nuclei or envelope of erythrocytes (Fig. 34); also seen touching nucleus or envelope of erythrocytes (Figs. 35–37), but usually not both these cellular structures at this stage of development. Advanced gametocytes slightly displace nuclei of erythro-

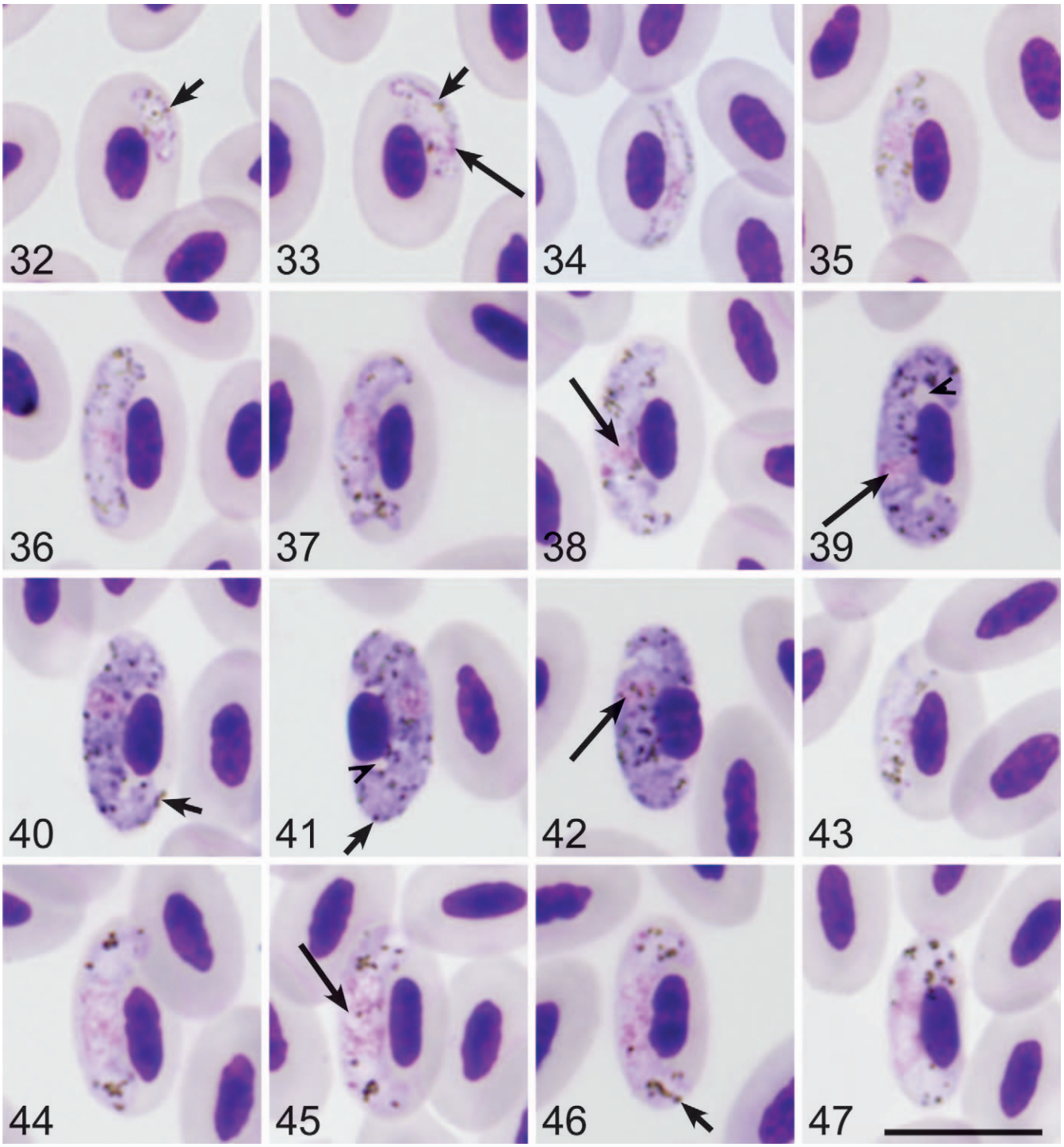


FIGURES 1–16. *Haemoproteus (Haemoproteus) multivolutinus* sp. nov. from the blood of the Tambourine dove *Turtur timpanistria*. (**1**) Young gametocyte. (**2–8**) Macrogametocytes. (**9–16**) Microgametocytes. Long simple arrows: nuclei of parasites. Long triangle arrows: vacuoles. Short arrows: pigment granules. Simple arrowheads: unfilled spaces between gametocytes and nuclei of infected erythrocytes. Triangle arrowheads: clumps of volutin. Giemsa-stained thin blood films. Bar = 10 μm.

cytes; usually touching both erythrocyte nuclei and envelope, filling erythrocytes up to their poles (Figs. 38–41). In advanced gametocytes, 2 clear unfilled spaces appear between the ends of gametocytes and nuclei of erythrocytes (Fig. 39), giving gametocytes a horn-like appearance, and disappearing as the parasite matures (Fig. 42). Mature gametocytes extend around the nuclei of erythrocytes, enclosing them with their ends, but do not encircle nuclei completely; they usually push nuclei with their middle part to envelope of erythrocytes (Figs. 39–41) and finally occupy nearly the entire cytoplasmic space in host cells. Fully grown gametocytes closely associated with nuclei and envelope of erythrocytes, filling erythrocytes up to their poles (Fig. 42). Parasite nucleus small (Table I), variable in form, frequently irregular in shape, submedian or sometimes median in position



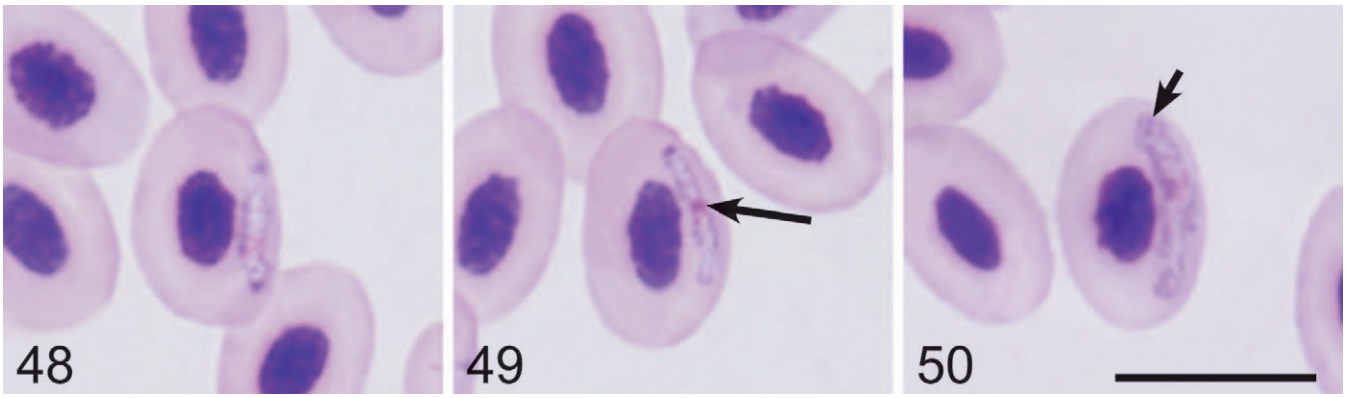
FIGURES 17–31. Mature gametocytes of widespread hippoboscid-transmitted species of hemoproteids. (**17–19**) *Haemoproteus columbae* from the blood of *Columba livia*; (**20–22**) *Haemoproteus sacharovi* from the blood of *Zenaida macroura;* (**23–25**) *Haemoproteus multipigmentatus* from the blood of *Zenaida galapagoensis*; (**26–28**) *Haemoproteus palumbis* from the blood of *Columba palumbus;* (**29–31**) *Haemoproteus turtur* from the blood of *Streptopelia turtur*. (17, 18, 20, 21, 23, 24, 26, 27, 29, 30) Macrogametocytes. (19, 22, 25, 28, 31) Microgametocytes. Simple long arrows: nuclei of parasites. Simple short arrows: pigment granules. Triangle arrow heads: clumps of volutin. Giemsa-stained thin blood films. Bar = 10 μm.



FIGURES 32–47. *Haemoproteus (Haemoproteus) paramultipigmentatus* sp. nov. from the blood of the Socorro common ground dove *Columbina passerina socorroensis*. (**32, 33**) Young gametocytes. (**34–42**) Macrogametocytes. (**43–47**) Microgametocytes. Long simple arrows: nuclei of parasites. Short simple arrows: pigment granules. Simple arrow heads: unfilled spaces between gametocytes and nuclei of infected erythrocytes. Giemsa-stained thin blood films. Bar = 10 μm.

(Figs. 38–42). Nucleolus not seen. Pigment granules of small size (<0.5 μm), roundish, black, numerous (Table I), randomly scattered throughout cytoplasm (Figs. 40, 41). Outline of gametocytes wavy (Fig. 38), slightly ameboid (Figs. 35, 37), or irregular (Figs. 39, 40), but more frequently the latter. Fully grown gametocytes are halteridial, they markedly displace nuclei of erythrocytes laterally (Fig. 42, Table I), but gametocytes in enucleated host cells not seen.

Microgametocytes (Figs. 43–47): General configuration as for macrogametocytes with usual hemosporidian sexually dimorphic characters. Pigment granules less numerous ($P < 0.05$) than in macrogametocytes (Table I).



FIGURES 48–50. Young gametocytes of *Haemoproteus (Haemoproteus) multipigmentatus* from the blood of the Galapagos dove *Zenaida galapagoensis*. (**48–50**) Note the markedly slender shape of earliest gametocytes. Long simple arrows: nuclei of parasites. Short simple arrows: pigment granules. Giemsa-stained thin blood films. Bar = 10 μm.

Taxonomic summary

Type host: Socorro common ground dove *Columbina passerina socorroensis* (Columbiformes, Columbidae).

Type locality: Socorro Island, Los Cedros, 18°45′28.9″N, 110°56′44.4″W.

Type specimens: Hapantotype (accession numbers 48658 NS, intensity of parasitemia is approximately 0.2%, lineage SocH3, GenBank JN788934, *C. passerina socorroensis*, collected by Jenny S. Carlson and Juan E. Martínez-Gómez, July 2009) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania (accession nos. 48659 NS, 48660 NS), in the U. S. National Parasite Collection, Beltsville, Maryland (USNPC 106052.00), and in the Queensland Museum, Queensland, Australia (G465621).

Additional material: One blood film (accession 48663, host is the Beautiful fruit dove (*Ptilinopus pulchelus*) samples from Papua New Guinea) is deposited in the CNRC, Vilnius.

DNA sequences: Mitochondrial cyt *b* lineages SocH3, SocH8 (694 base pairs, GenBank JN788934 and JN788939, respectively).

Site of infection: Mature erythrocytes; no other data.

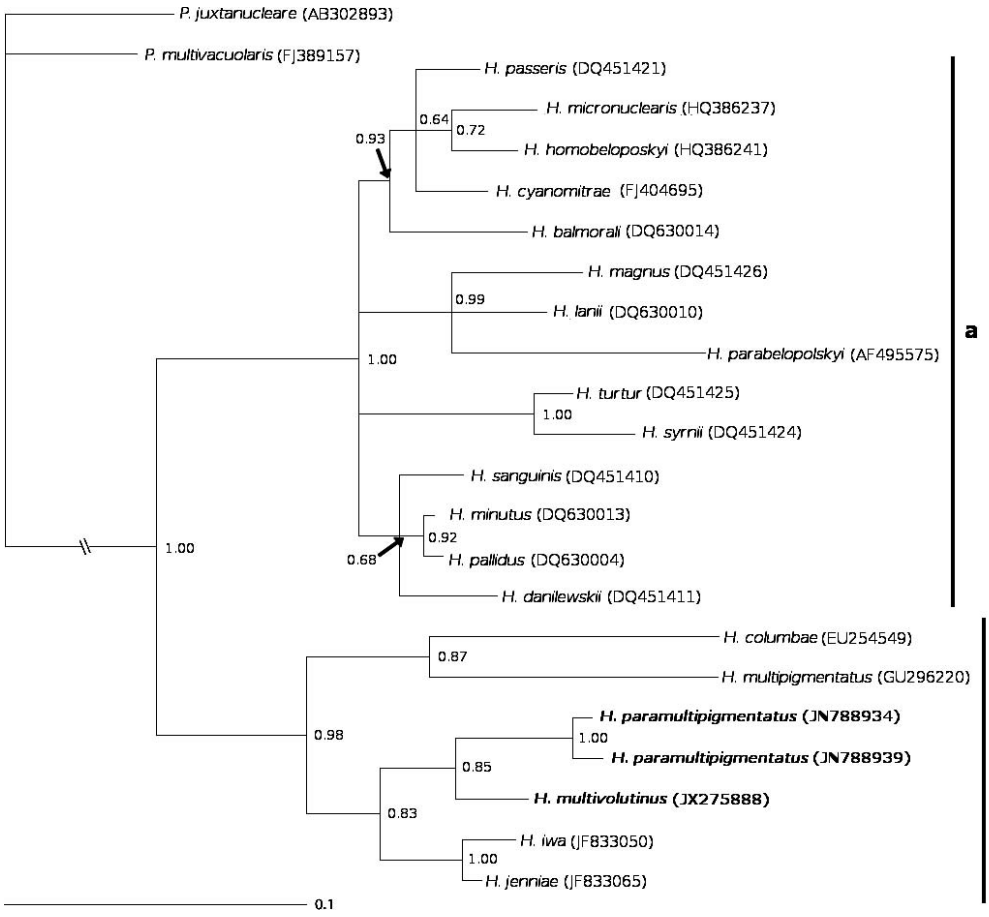


FIGURE 51. Bayesian majority-rule consensus phylogeny of 21 mitochondrial cytochrome *b* lineages of avian *Haemoproteus* spp. and 2 lineages of avian *Plasmodium* spp. used as an outgroup. GenBank accession numbers of sequences are given after parasite species names. Vertical lines indicate group of closely related lineages of hemoproteids belonging to the subgenera *Parahaemoproteus* (**a**) and *Haemoproteus* (**b**). Lineages in bold face represent parasite lineages of new species of hemoproteids. Values on branches represent the Bayesian posterior probabilities for the different nodes; scale bar is given in percentage.

Prevalence: Overall prevalence in the Socorro Islands was 10 of 23 (43.8%). In the type locality, the prevalence was 7 of 15 (46.7%).

Distribution and additional hosts: This parasite (the lineage SocH3 and gametocytes indistinguishable from parasites shown in Figs. 32–47) was reported in the Beautiful fruit dove in Papua New Guinea (T. A Iezhova, unpubl. obs.). Using BLAST with the SocH3 sequence revealed no identical sequences, but 5 similar sequences (GQ395639, JN788935, HM222487, GQ141567, and HM222486). These sequences differed by 4 pb (GQ395639, GQ141567, and HM222486), 15 bp (JN788935), and 19 bp (HM222487) when compared to SocH3. The lineage SocH3 differed from SocH8 by 7 bp. The sequences HM222487, GQ141567 and HM222486 were found in *C. passerina* while sequence JN788935 was found in *C. passerina socorrensis*. Sequence GQ395639 was isolated from the Galapagos penguin, *Spheniscus mendiculus*.

Etymology: The species name reflects the similarity of morphological features of gametocytes of this parasite to those of *H. multipigmentatus*, a common parasite of the endemic Galapagos dove.

Remarks

Among the six species of hemoproteids that parasitize doves and pigeons (see the Remarks to *H. multivolutinus* and Figs. 17–31), *H. paramultipigmentatus* is particularly similar to *H. multipigmentatus* (Figs. 23–25) due to its shape and mode of growth of gametocytes; this is reflected in the new species name. However, fully grown gametocytes of the new species possess approximately half of the pigment granules ($P < 0.01$) as compared to *H. multipigmentatus*. These species are also readily distinguishable at the stage of young gametocytes: the earliest forms are broadly ovoid in *H. paramultipigmentatus*, but are elongate slender bodies in *H. multipigmentatus* (compare Figs. 32 and 33 with Figs. 48 and 49). Advanced young gametocytes of these 2 species are similar in shape (compare Figs. 34 and 50).

It is straightforward to distinguish *H. paramultipigmentatus* from other hemoproteids of columbiform birds: gametocyetes of *H. multivolutinus* (Figs. 1–16), *H. columbae* (Figs. 17–19), *H. palumbis* (Figs. 26–28), and *H. turtur* (Figs. 29–31) possess prominent volutin; gametocytes of *H. sacharovi* possess a few fine pigment granules and are outwardly similar to gametocytes of *Leucocytozoon* spp. (Figs. 20—22). None of these features is characteristic of *H. paramultipigmentatus*.

Phylogenetic relationships of parasites

All identified species of avian hemoproteids are clearly distinguishable in the phylogenetic tree (Fig. 51), which corresponds with their morphological differences. Because parasites of 2 cyt *b* gene lineages of *H. paramultipigmentatus* in the Socorro common ground dove are closely related (Fig. 51, clade b) and are indistinguishable based on morphology of their gametocytes, we consider these 2 lineages as intraspecies genetic variation of the same morphospecies. Genetic distance between these lineages of *H. paramultipigmentatus* is 2.5%.

Genetic distance in cyt *b* gene between 2 lineages of *H. paramultipigmentatus* and 1 lineage of *H. multivolutinus* (see Fig. 51) is 4.7% and 5.8%.

Haemoproteus multivolutinus and *H. paramultipigmentatus* are attributed to the subgenus *Haemoproteus* because cyt *b* lineages of this parasite cluster well with the lineages of hippoboscid-transmitted *H. columbae, H. iwa, H. multipigmentatus* and *H. jenniae* (Fig. 51, clade b), but not to the lineages of other avian species of the subgenus *Parahaemoproteus* (Fig. 51, clade a). The genetic distance among 2 lineages of *H. paramultipigmentatus* and the lineages of the closely related hippoboscid-transmitted *H. columbae*, *H. multipigmentatus, H. iwa* and *H. jenniae* (Fig. 51, clade b) ranges between 2.5% and 12.4% (on average 7.9%). Genetic differences among 2 lineages of *H. paramultipigmentatus* and the lineages of less closely related ceratopogonid-transmitted *Haemoproteus (Parahaemoproteus)* spp. (Fig. 51, clade a) is greater; it ranges between 10.9% and 15.9% (on average 12.6%).

The genetic distance between the lineage of *H. multivolutinus* and the lineages of the closely related hippoboscid-transmitted *H. columbae*, *H. iwa*, and *H. jenniae* ranges between 4.7% and 9.2% (on average 6.4%). Genetic differences among the lineage of *H. multivolutinus* and the lineages of less closely related ceratopogonid-transmitted *Haemoproteus* (*Parahaemoproteus*) spp. (Fig. 33, clade a) is greater; it ranges between 10.5% and 14.2% (on average 12.0%).

DISCUSSION

Recent investigations reveal that some species of avian hemoproteids cause pathology in birds (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002) and are sometimes lethal (Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011). These parasites affect host fitness (Nordling et al., 1998; Marzal et al., 2005; Valkiūnas, 2005; Møller and Nielsen, 2007), and thus warrant more fundamental research and attention in conservation projects. Studies on the diversity and distribution of *Haemoproteus* parasites are important to better understand wildlife diseases, particularly the virulence and mortality caused by these pathogens both in avian hosts (Ferrell et al., 2007; Olias et al., 2011) and blood-sucking insects (Valkiūnas and Iezhova, 2004), which are poorly understood issues.

It worth noting that one of the parasites was found in two very different and distant locations. *Haemoproteus paramultipigmentatus* was found in doves of Socorro Island, but also in a Beautiful fruit dove from Papua New Guinea. It is not uncommon to find *Haemoproteus* species and their transmission in different regions of the planet, as is exhibited by the common dove parasites *H. columbae* and *H. sacharovi* (Valkiūnas, 2005), but it is surprising that this parasite was not described earlier, in particular since it is so morphologically distinct. It is possible that this parasite may have a tropical distribution and was overlooked due to lack of sampling.

Recent combined microscopic and PCR-based studies show that phylogenies based on partial sequences of mitochondrial cyt *b* gene distinguish hemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus*, which form distinct clades in the phylogenetic trees (Valkiūnas et al., 2010; Iezhova et al., 2011; Levin et al., 2012). This is not unexpected because parasites of these 2 subgenera are transmitted by different dipteran vectors (species of Hippoboscidae and Ceratopogonidae, respectively); and they undergo markedly different sporogony in the vectors (see Bennett et al., 1965; Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005; Santiago-Alarcón et al., 2012). Phylogenies based on cyt *b* sequences can be recommended for predicting possible vectors and thus provide valuable information to plan future vector studies of hemosporidian species, which vectors are unknown. Based on our phylogenetic analysis, hippoboscid flies should be incriminated as vectors of both *H. multivolutinus* and *H. paramultipigmentatus* because these parasites fall into the clade of the hippoboscid-transmitted hemoproteids (Fig. 51, clade b) and are more similar in cyt *b* gene to the species of the subgenus *Haemoproteus*. It is worth mentioning that *H.* (*Haemoproteus*) *turtur*, a common parasite of doves of the Old World, appeared in the *Parahaemoproteus* clade in different phylogenies of avian hemosporidians (Martinsen et al., 2008; Valkiūnas et al., 2010; see Fig. 51, clade a). Because all investigated parasites from the clade A are transmitted by biting midges (Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005), it might be that *H. turtur* is not transmitted by hippoboscid flies. The limited available information that hippoboscids transmit *H. turtur* (Rashdan, 1998) needs re-evaluation. This study adds *H. multivolutinus* and *H. paramultipigmentatus* to the phylogenetic studies of the possible hippoboscid-transmitted hemoproteids.

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