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A NEW *HAEMOPROTEUS* SPECIES (HAEMOSPORIDA: HAEMOPROTEIDAE) FROM THE ENDEMIC GALAPAGOS DOVE *ZENAIDA GALAPAGOENSIS*, WITH REMARKS ON THE PARASITE DISTRIBUTION, VECTORS, AND MOLECULAR DIAGNOSTICS

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ABSTRACT: *Haemoproteus (Haemoproteus) multipigmentatus* n. sp. (Haemosporida, Haemoproteidae) was found in the endemic Galapagos dove *Zenaida galapagoensis*. It is described based on the morphology of its blood stages and segments of the mitochondrial cytochrome *b* gene, which can be used for molecular identification and diagnosis of this species. *Haemoproteus multipigmentatus* can be readily distinguished from all species of hemoproteids of the subgenus *Haemoproteus*, primarily due to numerous (approximately 40 on average) small pigment granules in its mature gametocytes. Illustrations of blood stages of the new species are given, and phylogenetic analysis identifies DNA lineages closely related to this parasite, which is prevalent in the Galapagos dove and also has been recorded in other species of Columbiformes in Mexico, Guatemala, and Peru, and so seems to be widespread in countries in the New World with warm climates. Cytochrome *b* lineages of *H. multipigmentatus* cluster with hippoboscid-transmitted lineages of *Haemoproteus columbae*. The same lineages of *H. multipigmentatus* were recorded in thoraxes of the hippoboscid fly *Microlynchia galapagoensis*, which likely is a natural vector of this parasite in Galapagos. Because different primers might amplify different parasites if they have a better match during a simultaneous infection, it is important that researchers standardize the genetic marker of choice for molecular typing of hemosporidian species. This study shows that more discussion among researchers is needed to clearly establish the sequence length and number of genes used for identification of hemosporidian parasites at different taxonomic levels. We point to the need of using both morphology and gene markers in studies of hemosporidian parasites, particularly in wildlife.

During an ongoing study on the distribution and evolutionary biology of pathogens in Galapagos (Padilla et al., 2004; Parker et al., 2006; Santiago-Alarcon et al., 2008; Levin et al., 2009; Santiago-Alarcon et al., 2010), blood samples and hippoboscid flies (Hippoboscidae) were collected from the endemic Galapagos dove *Zenaida galapagoensis* and other columbiform birds in the New World between 2002 and 2009. One previously undescribed species of *Haemoproteus* (Haemosporida, Haemoproteidae) was found during this study. This parasite is described here, using data on the morphology of its blood stages and partial sequences of the mitochondrial cytochrome *b* (cyt *b*) gene. We also identify a probable vector of this hemoproteid in the Galapagos archipelago and generalize available information about its distribution and avian host range. Some problems of molecular identification and diagnostics of hemosporidian parasites using partial DNA sequences are also discussed.

MATERIAL AND METHODS

Collection of blood samples and hippoboscid flies

In all, 443 blood samples were collected from doves and pigeons in North and South America and the West Indies between 2002 and 2009. The birds were caught with mist nets and hand nets. We collected 170 blood samples from Galapagos doves on 10 islands of the Galapagos archipelago (Santiago, Santa Cruz, Santa Fe, Española, San Cristobal, Genovesa, Marchena, Fernandina, Darwin, and Wolf). Blood samples were also obtained from 17 species of columbiform birds belonging to 7 genera in the United States (2 samples), Mexico (7), Caribbean islands (10), Venezuela (126), Peru (29), Uruguay (2), Ecuador (73), and Guatemala (10). Samples from Ecuador (Galapagos and the mainland), Peru, and the United States were collected by the authors. Samples from other localities were provided to us by colleagues (for details about study sites and investigated bird species, see Santiago-Alarcon et al., 2010). For a description of the new species of parasite, samples from 10 Galapagos doves and 3 continental species of Columbiformes were used; these samples were selected based on the availability and quality of blood smears for morphological work and on the close similarity among Galapagos and mainland parasite lineages, as identified by Santiago-Alarcon et al. (2010).

Blood was taken by puncturing the brachial vein; all birds were then released, with none of the individuals being recaptured. Approximately 50 μl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were preserved in lysis buffer (Longmire et al., 1988) and then held at ambient temperature in the field and later at −20 C in the laboratory.

Blood smears were collected only from Galapagos doves. Blood films were air-dried within 5–10 sec after their preparation; they were fixed in absolute methanol in the field and then stained with Quick Field's stain (2002–2008 samples) and with Giemsa (2009 samples) in the laboratory. Blood films were examined for 10–15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1,000). Detailed protocols of preparation, fixation, staining, and microscopic examination of blood films are described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light, i.e., <0.1%, as recommended by Godfrey et al. (1987). To determine the possible presence of simultaneous infections with other hemosporidian parasites in the type material of new species, all blood films from hapantotype and parahapantotype series were examined microscopically at low magnification.

Hippoboscid flies *Microlynchia galapagoensis* were collected by hand during bird manipulation, directly from the plumage of Galapagos doves. The insects were stored in 95% alcohol in the field and later at 4 C in the laboratory until DNA extraction and subsequent testing using polymerase chain reaction (PCR). Seven individual flies were used in this study.

Morphological analysis

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, prepare illustrations, and take measurements. The morphometric features studied (Table I) are those defined by Valkiūnas (2005). Morphology of the 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 rock dove *Columba livia*, accession nos. 2905.87, 47723 NS, and 47724 NS in the Collection of Institute of Ecology, Nature Research Centre [CNRC]), *Haemoproteus sacharovi* (Mourning dove *Zenaida macroura*, nos. 45236A, 45236B, and 103700 in the Queensland museum, Queensland, Australia, and no. 47739 in the CNRC), *Haemoproteus turtur* (Turtle dove *Streptopelia turtur*, no. 1315.87 in the

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CNRC), and *Haemoproteus palumbis* (Woodpigeon *Columba palumbus*, 969, 970 in the Natural History Museum, London, United Kingdom, and no. 2067.87 in the CNRC). Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A *P* value of 0.05 or less was considered significant.

DNA extraction, PCR amplification, and sequencing

Infections were determined by microscopic examination of blood smears and by PCR amplification of parasite gene sequences. DNA was extracted using the phenol-chloroform method followed by dialysis in 1× TNE_2 (Sambrook and Russell, 2001). Published primers and protocols from Waldenström et al. (2004) were used to amplify a fragment of the parasites' mitochondrial cytochrome *b* (cyt *b*) gene. PCR products were cleaned directly using Antarctic phosphatase and Exonuclease I (M0289S and M0293S respectively, New England Bio Labs, Inc., Ipswich, Massachusetts). We used an ABI PRISM® 3100 microcapillary genetic analyzer (Applied Biosystems, Life Technologies, Carlsbad, California) to sequence DNA products. Sequences were edited in 4Peaks v1.7.2 (2005, http://mekentosj.com/science/4peaks/) and aligned by eye in Se-Al v2.0a11 (1996–2002, http://tree.bio.ed.ac.uk/software/seal/). New sequences were deposited in GenBank (accession numbers: GU296210–GU296227).

In the laboratory, thoraxes of 7 hippoboscid flies *M. galapagoensis* were carefully severed from the heads and abdomens. Each thorax was used for DNA extraction; we used a Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, California). The standard protocol was followed; however, DNA was eluted in half with buffer due to assumed low concentrations of any parasite DNA. Protocols for PCR amplification and sequencing were as described above.

To ensure that the positive PCR results from insects were DNA from sporozoites and not from some undigested parasite-infected blood cells that might have persisted in the vector digestive system as remnants of a blood meal, thoraxes of all insects were tested for bird mitochondrial cyt *b* gene with primers and protocols used by Ngo and Kramer (2003). Galapagos dove mitochondrial DNA was used as a positive control to identify and compare bird DNA amplified from insect thoraxes.

Phylogenetic analysis

The phylogenetic history of the new species and related hemosporidian parasites was reconstructed by using sequence information from our former studies and GenBank for the mitochondrial cyt *b* gene. Because GenBank contains information about numerous incorrectly identified species of hemosporidians (see Valkiūnas, Atkinson et al., 2008), we used mainly sequences of positively identified avian parasites (for examples of linking parasite lineages with their morphospecies, 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, Atkinson et al., 2008; Valkiūnas, Iezhova, Loiseau et al., 2008; Svensson and Ricklefs, 2009; Valkiūnas et al., 2009; Iezhova et al., 2010).

Phylogenetic hypotheses were constructed using the program Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist, 2001). We performed 3 independent runs, with 4 chains in each run, for a total of 3 million generations, sampling every 100 generations. The first 15,000 trees were discarded as the "burn-in" periods. In total, 15,000 trees from each run were used to build our majority-rule consensus tree. For the analyses, we used a GTR+I+Γ model of molecular evolution with shape parameter $\alpha = 0.45$, and proportion of invariable sites Pinvar = 0.34 as calculated from the data using Mr. Bayes v3.1.2.

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

Table I. Morphometry of host cells and mature gametocytes of *Haemoproteus multipigmentatus* sp. nov. from the Galapagos dove *Zenaida galapagoensis*.

Feature	Measurements (μm)*
Uninfected erythrocyte	
Length	10.5–12.2 (11.3 ± 0.5)
Width	6.4–7.9 (7.1 ± 0.3)
Area	58.2–72.0 (64.1 ± 4.0)
Uninfected erythrocyte nucleus	
Length	4.1–6.1 (5.2 ± 0.5)
Width	2.1–2.9 (2.5 ± 0.2)
Area	9.5–13.2 (11.0 ± 1.1)
Macrogametocyte	
Infected erythrocyte	
Length	11.5–14.5 (13.1 ± 0.9)
Width	4.9–7.3 (6.5 ± 0.5)
Area	59.6–76.7 (69.6 ± 5.3)
Infected erythrocyte nucleus	
Length	4.4–5.9 (5.2 ± 0.4)
Width	2.3–3.2 (2.8 ± 0.3)
Area	10.1–14.6 (12.0 ± 1.2)
Gametocyte	
Length	13.7–20.4 (16.4 ± 1.6)
Width	2.6–4.1 (3.3 ± 0.4)
Area	37.7–54.8 (47.8 ± 5.3)
Gametocyte nucleus	
Length	2.0–2.9 (2.4 ± 0.2)
Width	1.2–2.2 (1.7 ± 0.3)
Area	2.5–4.4 (3.4 ± 0.5)
Pigment granules	33.0–54.0 (43.4 ± 5.2)
NDR†	0.0–0.7 (0.2 ± 0.2)
Microgametocyte	
Infected erythrocyte	
Length	11.1–14.1(13.1 ± 0.8)
Width	5.8–7.6 (6.7 ± 0.5)
Area	54.3–80.8 (70.3 ± 7.0)
Infected erythrocyte nucleus	
Length	4.7–5.7 (5.2 ± 0.2)
Width	2.2–3.3 (2.6 ± 0.3)
Area	8.7–13.1 (11.0 ± 1.0)
Gametocyte	
Length	12.4–16.2 (14.6 ± 0.9)
Width	2.7–3.9 (3.3 ± 0.4)
Area	42.1–55.3 (48.4 ± 4.7)
Gametocyte nucleus	
Length	5.1–9.2 (6.3 ± 1.0)
Width	1.7–3.1 (2.6 ± 0.4)
Area	9.9–18.3 (14.9 ± 2.4)
Pigment granules	30.0–48.0 (38.9 ± 5.0)
NDR	0.0–0.6 (0.4 ± 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 ratio according to Bennett and Campbell (1972).

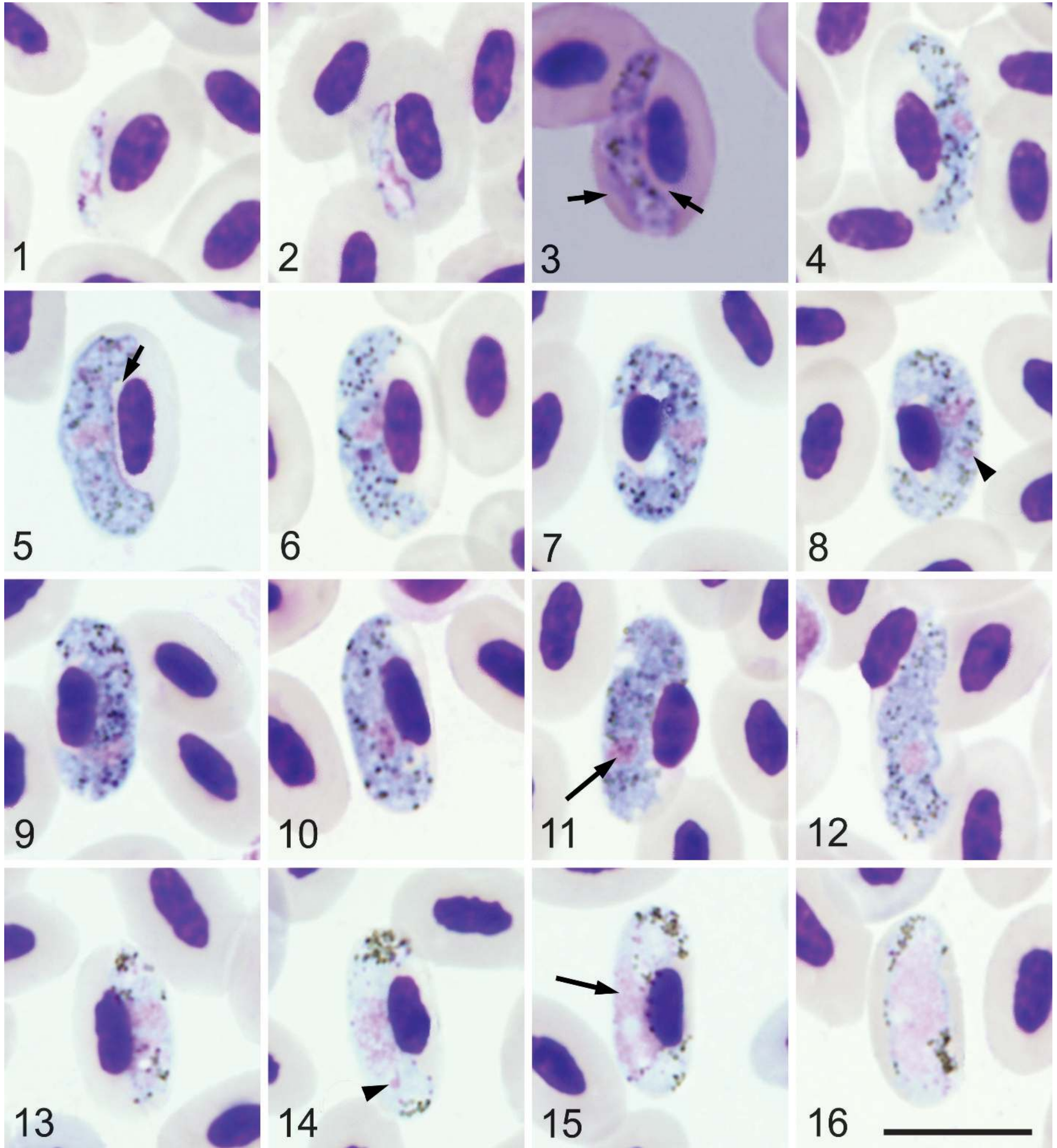
RESULTS

Haemoproteus (Haemoproteus) multipigmentatus n. sp.

(Figs. 1–16, Table I)

Description

Young gametocytes (Figs. 1–2): Develop in mature erythrocytes. Earliest forms seen anywhere in infected erythrocytes, but more frequently recorded lateral to erythrocyte nuclei; markedly variable in shape. With development, gametocytes extend along nuclei of erythrocyte, touching neither nuclei nor envelope of erythrocytes (Fig. 1). Pigment granules small (<0.5 μm), black, and frequently grouped (Fig. 2). A few roundish, light-violet,



FIGURES 1–16. *Haemoproteus (Haemoproteus) multipigmentatus* sp. nov. from the blood of the Galapagos dove *Zenaida galapagoensis*. (**1, 2**) Young gametocytes. (**3–12**) Macrogametocytes. (**13–16**) Microgametocytes. Long arrows, nuclei of parasites. Short arrows, unfilled spaces among gametocytes and envelope and nuclei of infected erythrocytes. Arrow heads, azurophilic granules. (1, 2, 4–16) Giemsa-stained thin blood films. (3) Field-stained thin blood films. Bar = 10 μm.

small volutin granules usually present. Outline of growing gametocytes wavy (Fig. 1), irregular (Fig. 2), or slightly ameboid. Influence of young gametocytes on infected erythrocytes usually not pronounced.

Macrogametocytes (Figs. 3–12): Extend along nuclei of erythrocytes; elongate slender bodies with wavy, irregular, or slightly ameboid outline. Cytoplasm blue, homogeneous in appearance, usually possesses small (<0.5 μm), light-violet volutin granules

and few vacuoles; small (<1 μm in diameter), azurophilic granule frequently seen (Fig. 8). Growing gametocytes, with length exceeding length of erythrocyte nuclei (Figs. 3–5), 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. 3); also seen touching nucleus or envelope of erythrocytes (Figs. 4, 5), but usually not both of these cellular structures at this stage of development. Advanced gametocytes do not displace or only slightly displace nuclei of erythrocytes; usually in touch with both erythrocyte nuclei and envelope, filling erythrocytes to their poles (Fig. 6). Mature gametocytes extend around nuclei of erythrocytes, enclosing them with their ends, but do not encircle nuclei completely (Figs. 7, 8); they usually push nuclei with their middle part to envelope of erythrocytes (Fig. 7) and finally occupy nearly the entire cytoplasmic space in host cells (Fig. 9). In advanced gametocytes, 2 clear, unfilled spaces appear between ends of gametocytes and nuclei of erythrocytes (Figs. 7, 8), giving gametocytes horn-like appearance, and disappearing as parasite matures (Figs. 9–11). Fully grown gametocytes closely associated with nuclei and envelope of erythrocytes, filling erythrocytes to their poles (Figs. 9–11). Parasite nucleus small (Table I), variable in form, frequently irregular in shape, median or submedian in position (Figs. 4–12). Nucleolus frequently seen. Pigment granules small (<0.5 μm), roundish, black, numerous (Table I), randomly scattered throughout cytoplasm. Outline of gametocytes irregular (Figs. 4, 6, 12), wavy (Figs. 7, 8), or slightly ameboid (Figs. 9–11), but more frequently the latter. Mature gametocytes are halteridial, they markedly displace nuclei of erythrocytes laterally (Figs. 9, 10), frequently to envelope of erythrocytes (Fig. 11); such gametocytes predominate in type material. Fully grown gametocytes markedly displace nuclei of infected erythrocytes, sometimes asymmetrically (Fig. 10), and even to poles of erythrocytes (Fig. 12). Gametocytes in enucleated host cells present in all type preparations, but rare in number (<1% of all gametocytes).

Microgametocytes (Figs. 13–16): General configuration as for macrogametocytes, with usual haemosporidian sexually dimorphic characters. Pigment granules lighter in color than in macrogametocytes, gathering close to ends of gametocytes. Enucleated host cells present (Fig. 16) with same frequency as for macrogametocytes.

Vector studies

Three closely related lineages (hHIPP26W, hHIPP28W, hHIPP30W, see Fig. 33, Box B), which are identical or closely related to lineages of *H. multipigmentatus* recorded in birds, were found in the thoraxes of 3 hippoboscid flies *M. galapagoensis* collected from Galapagos doves on Santiago Island, Santa Fe Island, and Española Island. Because the thoraxes of these flies were PCR-positive for parasite DNA, but negative for bird DNA, it is likely that the detected parasite lineages are not from intraerythrocytic gametocytes, but belong to the sporozoite stage of *H. multipigmentatus*. Additionally, 1 thorax was positive for both parasite (lineage hHIPP29W, Fig. 33, Box B) and bird DNA, 2 thoraxes were negative for parasite but positive for bird DNA, and 1 was negative for both parasite and bird DNA. We compared the bird cyt *b* sequences obtained from fly thoraxes to what is available in GenBank by using the BLAST algorithm. Our results showed similarities (best match) of 98–100% to a cyt *b* sequence obtained from a Galapagos dove (accession number AF251531), showing that insects certainly feed on the doves. These data show that *M. galapagoensis* is a probable natural vector of *H. multipigmentatus*.

Taxonomic summary

Type host: Zenaida galapagoensis Gould (Columbiformes, Columbidae).

Type locality: Cueva Norte, Fernandina, Galapagos, Ecuador (0°28.166′S, 91°50.899′W, approximately 30 m above sea level).

Type specimens: Hapantotype (accession numbers 47725 NS, 47726 NS, intensity of parasitemia is 0.1%, *Z. galapagoensis*, Cueva Norte, Fernandina, Galapagos, 00°28.166′S, 91°50.899′W, lineage hJH003W, collected by G. Valkiūnas, 18 July 2009) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accession nos. USNPC 102680, USNPC 102681; G465418, G465419; and 47727 NS, 47728 NS) are deposited in the U.S. National Parasite Collection, Beltsville, Maryland; in the Queensland Museum, Queensland, Australia; and in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, respectively.

Additional material: Two blood films (accession numbers USNPC 102682, G465420, intensity of parasitemia is 0.01%, *Z. galapagoensis*, Santa Cruz, Charles Darwin Station, 00°44.338′S, 90°18.108′W, collected by P. G. Parker, 10 July 2009) are deposited in the U.S. National Parasite Collection, Beltsville, Maryland, and in the Queensland Museum, Queensland, Australia, respectively.

DNA sequences: Mitochondrial cyt *b* lineages hJH003W, hJH3B002W, and hJH3008W from type material (481, 492, and 481 base pairs, respectively; GenBank accession nos. GU296216, GU296215, and GU296224, respectively).

Site of infection: Mature erythrocytes; no other data.

Vector: Microlynchia galapagoensis (Diptera, Hippoboscidae) is a probable vector in Galapagos.

Prevalence: In the type locality, the prevalence was 3 of 3 (100%). Overall prevalence in the Galapagos dove in different islands in Galapagos ranges between 36% and 100% (Padilla et al., 2004; Santiago-Alarcon et al., 2008).

Distribution and additional hosts: The lineages hLPMEXW, hCTGUA1W, and hZA16PERUW have been recorded in columbiform birds in Mexico (host is Grey-headed dove *Leptotila plumbeiceps*), Guatemala (ruddy ground-dove *Columbina talpacoti*), and Peru (eared dove *Zenaida auriculata*), respectively. These lineages are closely related to the lineages of *H. multipigmentatus* from the parasite's type material (Fig. 33, Box B). *Haemoproteus multipigmentatus* is widely distributed throughout the range of the Galapagos dove in Galapagos and also is transmitted among other species of Columbiformes in countries in the New World with warm climates.

Etymology: The species name reflects the presence of numerous pigment granules in mature gametocytes of this parasite.

Remarks

Six species of hemoproteids parasitize birds belonging to Columbiformes (Bennett and Peirce, 1990; Valkiūnas, 2005).

Haemoproteus maccallumi Novy and MacNeal, 1904 was also described in columbiform birds. However, the original description of this parasite is based on simultaneous infection of *H. columbae* and *H. sacharovi*, so the name *H. maccallumi* is a partial synonym of both of these parasites and thus is invalid (see Novy and MacNeal, 1904; Valkiūnas, 2005). *Haemoproteus multipigmentatus* can be readily distinguished from all of these parasites based on the numerous (approximately 40 in average) pigment granules in its mature gametocytes (Table I, Figs. 4–16).

Four species of hemoproteids parasitize doves and pigeons (Figs. 17–32): *H. columbae* (Kruse, 1890), *H. palumbis* (Baker, 1966), *H. sacharovi* (Novy and MacNeal, 1904), and *H. turtur* (Covaleda Ortega and Gállego Berenguer, 1950), and so should be distinguished from *H. multipigmentatus*. All of these parasites are transmitted by hippoboscid flies and belong to the subgenus *Haemoproteus* (Bennett et al., 1965; Atkinson, 1991; Valkiūnas, 2005). In addition to the number of pigment granules, *H. multipigmentatus* 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. 21, 22), which frequently exceed 1 μm in diameter in microgametocytes (Figs. 23, 24). Mature gametocytes of *H. sacharovi* are highly pleomorphic and possess fine pigment granules (Figs. 29–32), and are outwardly similar to gametocytes of *Leucocytozoon* spp.; average width of fully grown gametocytes of this parasite is >5 μm (Valkiūnas, 2005). Mature gametocytes of *H. palumbis* do not displace or only slightly displace nuclei of infected erythrocytes (Figs. 25–28). None of these features is characteristic of *H. multipigmentatus*, which is particularly similar to *H. turtur*, so should be compared with the latter parasite. Fully grown gametocytes of *H. turtur* usually do not touch the nuclei of erythrocytes (Figs. 17–20); they frequently possess slightly elongated medium-size (0.5–1 μm) pigment granules and are overfilled with prominent volutin gathered mainly on the ends of the parasites (see Figs. 17–20); these features are not characteristic of *H. multipigmentatus*. Additionally, based on material from type vertebrate hosts, area of macrogametocyte nuclei in *H. multipigmentatus* is approximately half the size of those in *H. turtur* ($P < 0.001$).

Phylogenetic relationships of parasites

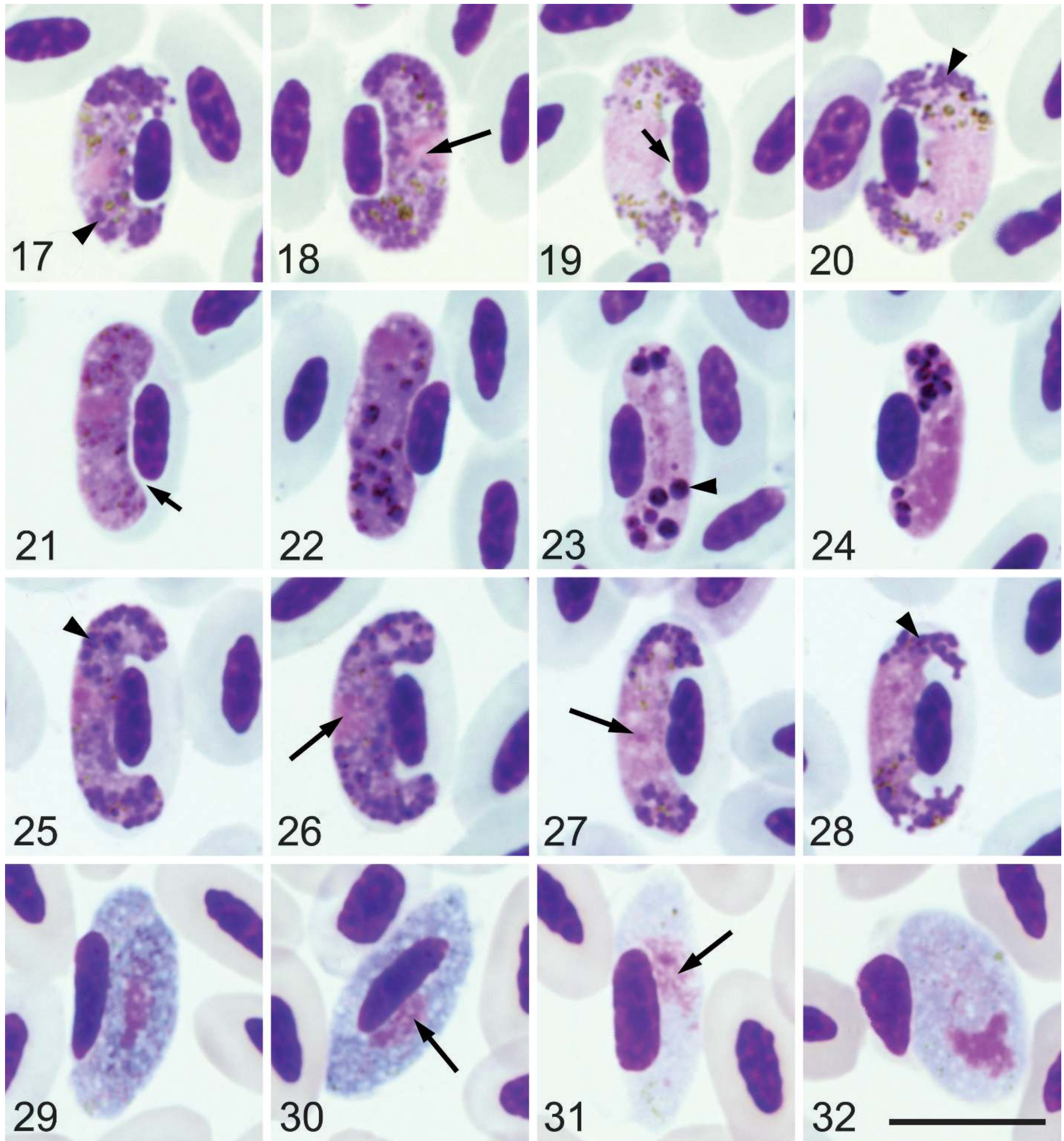
All positively identified species of avian hemoproteids are clearly distinguishable in the phylogenetic tree (Fig. 33), which corresponds with their morphological differences. Because parasites of the lineages recorded in the type material of *H. multipigmentatus*, as well as all other lineages of hemoproteids in the Galapagos dove, are closely related (Fig. 33, Box B) and are indistinguishable based on morphology of their blood stages, we consider all of these lineages as intraspecies genetic variation of the same morphospecies, i.e., *H. multipigmentatus*.

Genetic distance in the cyt *b* gene among different lineages of *H. multipigmentatus* ranges between 0.2% and 3.9%, and it is <2.5% for the great majority of lineages of this parasite (Fig. 33, Box B). Genetic distance between all recorded lineages of *H. multipigmentatus* and the lineages of hippoboscid transmitted *H. (Haemoproteus) columbae* ranges between 7.5% and 10.6%. Genetic differences among lineages of *H. multipigmentatus* and the lineages of positively identified species of ceratopogonid transmitted *Haemoproteus (Parahaemoproteus)* spp. (Fig. 33, Box A) ranges between 8.6% and 15.7%.

DISCUSSION

Haemoproteus multipigmentatus is assigned to the subgenus *Haemoproteus* because of 2 sets of our data. First, cyt *b* lineages of this parasite cluster well with the lineages of *H. (Haemoproteus) columbae* (Fig. 33, Box B), but not with the lineages of other avian species of the subgenus *Parahaemoproteus* (Fig. 33, Box A). Hemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of dipteran vectors (species of Hippoboscidae and Ceratopogonidae, respectively). In addition, they undergo markedly different sporogony in the vectors (see Bennett et al., 1965; Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005), and so usually appear in different well-supported clades in phylogenetic trees (Martinsen et al., 2008; Santiago-Alarcon et al., 2010; Iezhova et al., 2010). Second, the same and closely related lineages of *H. multipigmentatus* were also detected in thoraxes of hippoboscid flies *M. galapagoensis*, which were collected from Galapagos doves. Because the thoraxes of 3 flies were PCR-positive for parasite DNA but negative for avian DNA, these lineages likely belong to sporozoite stage of *H. multipigmentatus*. In avian hemosporidians, sporozoites represent the only sporogonic stage, which are present in the thoraxes of dipteran vectors, mainly in salivary glands (Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005). It is important to note that biting midges, vectors of *Haemoproteus (Parahaemoproteus)* species, were not collected in mosquito traps at the type locality of *H. multipigmentatus* (G. Valkiūnas, unpubl. obs.); this is a very dry desert site. The traps were covered with a fine mesh and were satisfactory for catching biting midges. It is unlikely that biting midges, which require relatively high humidity for active life (Glukhova, 1989), are the vectors of this hemoproteid at this study site. It is most probable that *H. multipigmentatus* is transmitted by the hippoboscid fly *M. galapagoensis*, which is prevalent on the Galapagos dove and parasitizes this bird throughout the archipelago, including dry sites without permanent freshwater, as on Wolf Island (D. Santiago-Alarcon, unpubl. obs.). Thus, these results support the role of *M. galapagoensis* as the natural vector of *H. multipigmentatus* in the Galapagos archipelago. Detection of oocysts in mid-gut and sporozoites in salivary glands of the flies, ideally followed by experimental infection of uninfected doves by sporozoites, are needed to provide unequivocal support that *M. galapagoensis* is the vector.

It is still unclear whether the phylogenetic analysis of cyt *b* genes can be applied for molecular identification of the subgeneric position of all hemoproteid species. This is mainly because the phylogenetic position of the majority of hippoboscid-transmitted morphospecies of subgenus *Haemoproteus* remains unknown. Surprisingly, *H. (Haemoproteus) turtur*, a common parasite of doves in the Old World, appeared in the *Parahaemoproteus* clade in different phylogenies of avian hemosporidians (Martinsen et al., 2008; Santiago-Alarcon et al., 2010; see Fig. 33, Box A). Because this parasite completes sporogony in hippoboscid flies and belongs to the subgenus *Haemoproteus* (Rashdan, 1998; Valkiūnas, 2005), it might be that molecular identification of hippoboscid-transmitted hemoproteids using currently applied molecular markers cannot be employed for all species of these parasites. Sequences of other positively identified hemoproteids that are transmitted by hippoboscids, as well as additional sequences of *H. turtur*, are needed to clarify this issue. Further



FIGURES 17–32. Mature gametocytes of widespread hippoboscid-transmitted species of hemoproteids. (**17–20**) *Haemoproteus (Haemoproteus) turtur* from the blood of *Streptopelia turtur*; (**21–24**) *Haemoproteus (Haemoproteus) columbae* from the blood of *Columba livia*; (**25–28**) *Haemoproteus (Haemoproteus) palumbis* from the blood of *Columba palumbus*; (**29–32**) *Haemoproteus (Haemoproteus) sacharovi* from the blood of *Zenaida macroura*; (17, 18, 21, 22, 25, 26, 29, 30) Macrogametocytes; (19, 20, 23, 24, 27, 28, 31, 32) Microgametocytes. Long arrows, nuclei of parasites. Short arrows, unfilled spaces among gametocytes and nuclei of infected erythrocytes. Arrow heads, volutin granules. Giemsa-stained thin blood films. Bar = 10 μm.

work to increase the number of precise linkages between hemosporidian DNA lineages with their morphospecies, particularly of hippoboscid-transmitted parasites of the subgenus *Haemoproteus*, is important. This study adds *H. multipigmentatus* to the phylogenetic studies of the hippoboscid transmitted hemoproteids.

We used mainly positively identified morphospecies of avian hemoproteids in the phylogenetic analysis (Fig. 33). Genetic

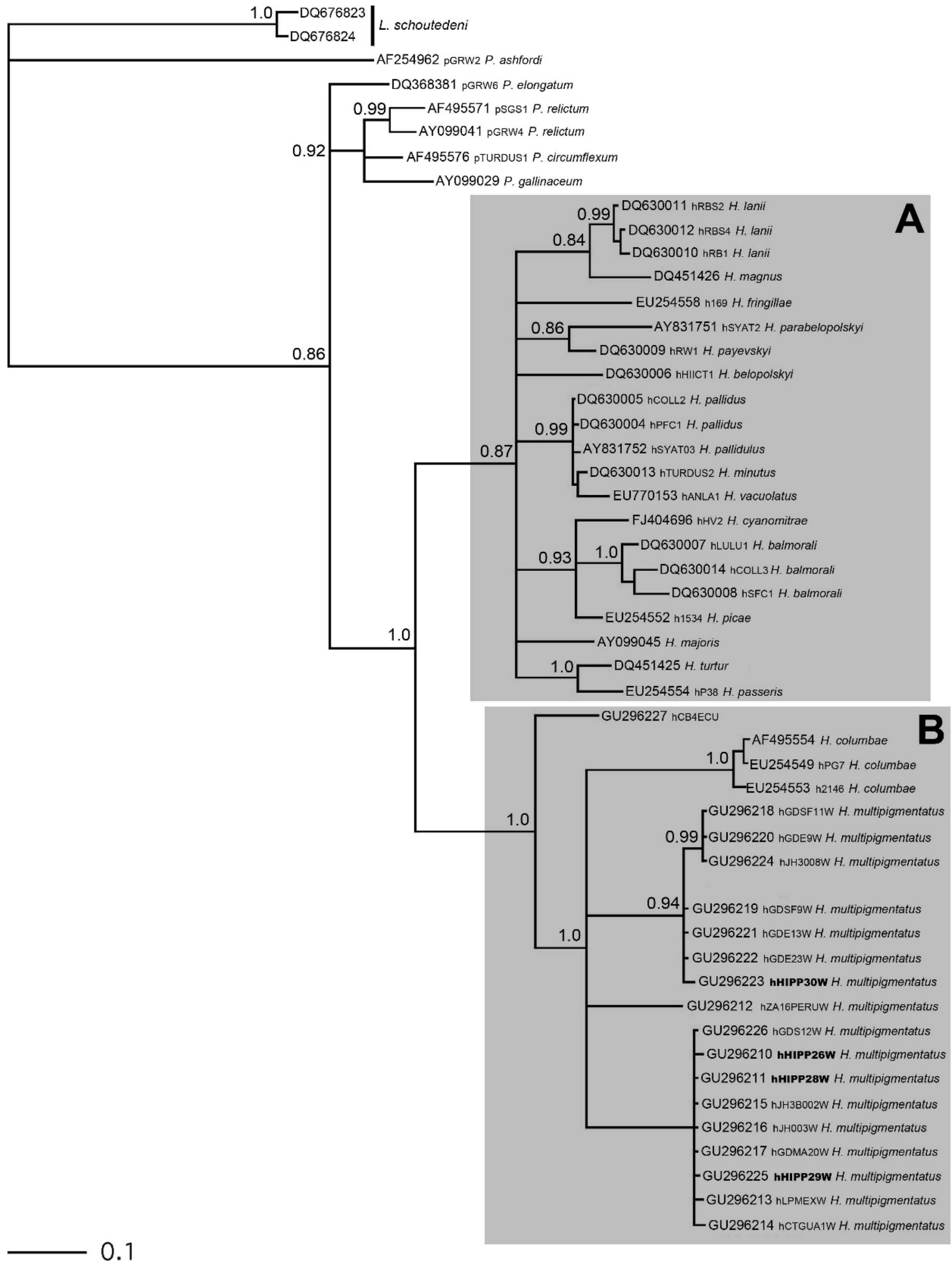


FIGURE 33. Bayesian majority-rule consensus phylogeny of 48 mitochondrial cytochrome *b* lineages of avian hemosporidians and 2 lineages of *Leucocytozoon shoutedeni* used as an outgroup. GenBank accession numbers of sequences and names of lineages are given before parasite species names. Gray boxes indicate group of closely related lineages of hemoproteids belonging to the subgenera *Parahaemoproteus* (**A**) and *Haemoproteus* (**B**). Lineages in bold face represent parasite lineages recovered from the hippoboscid fly *Microlynchia galapagoensis*, the probable vector of *Haemoproteus* (*H.*) *multipigmentatus* in the Galapagos archipelago. Values on branches represent the Bayesian posterior probabilities for the different nodes; scale bar is given in percentage.

distances between all cyt *b* lineages of *H. multipigmentatus* and the lineages of *H. columbae* is >7.5%. Genetic divergence among lineages of all positively identified morphospecies of hemosporidian parasites is >4%; it is >5% for the great majority of the readily distinguishable morphospecies (see Fig. 33), implying that genetic divergence of >5% can be used for the better understanding of phylogenetic trees based on the fragment of the cyt *b* gene used in the present study. This conclusion supports the hypothesis of Hellgren et al. (2007) that haemosporidian species with a genetic distance >5% in the mitochondrial cyt *b* gene are expected to be morphologically different. This has been shown to be true for many readily distinguishable morphospecies of avian hemosporidian parasites of the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (but see also Valkiūnas et al., 2009; Iezhova et al., 2010). Accumulation of information on this subject is useful because it provides more data for better understanding of phylogenetic trees based on a certain fragment of the cyt *b* gene.

It is interesting that the lineage hCB4ECU, which was obtained from the blood of an Ecuadorian ground dove *Columbina buckleyi* in mainland Ecuador, clusters with lineages of hemoproteids of the subgenus *Haemoproteus* (Fig. 33, Box B). Because genetic distance among the lineage hCB4ECU and other lineages of *H. multipigmentatus* and *H. columbae* is >7%, it is possible that the former lineage belongs to a different morphospecies. However, when parasite PCR products from the same sample were sequenced using the primers developed by Perkins and Schall (2002), which amplify the other section of the mitochondrial cyt *b* gene of hemoproteids, the lineage hCB4ECU is equal to a parasite lineage GDE9 obtained from the endemic Galapagos doves and is similar to several other parasite lineages retrieved from endemic Galapagos doves as well, e.g., lineages GDE23, GDMA20, and GDSF9 (see Santiago-Alarcon et al., 2010).

We think that this situation can be due to a possibly undetected mixed infection of hCB4ECU and *H. multipigmentatus* and primer bias when amplifying different sections of the cyt *b* gene of these parasites. PCR frequently does not read mixed hemosporidian infections (Valkiūnas et al., 2006), which are common in mainland birds, and different primers might amplify different parasites if they have a better match during a mixed infection with 2 or more related organisms (Cosgrove et al., 2006; Szöllősi et al., 2008). This issue could be settled if morphological material was available. Unfortunately, we do not have access to such information, which strongly points to the need of using both morphology and gene markers in studies of hemosporidian parasites, particularly in wildlife. Importantly, blood films, which are used for microscopic examination, should be prepared, stained, and examined properly (see Valkiūnas, Iezhova, Križanauskienė et al., 2008); this was not the case in some recent evolutionary biology studies. In addition, it is important for avian hemosporidian researchers to standardize the sequence length and genetic marker of choice for hemosporidian parasite identification. Until now, the primers of Waldenström et al. (2004) have been used successfully for this task. Moreover, it seems that when it comes to the mitochondrial cyt *b* gene, it does not matter whether longer or shorter fragments are used (Hellgren et al., 2007). However, the question of the lineage hCB4ECU raised here suggests that more discussion among researchers is needed to clearly establish the sequence length and number of genes used for identification of hemosporidian parasites at different taxonomic levels.

All recorded lineages of *H. multipigmentatus* (Fig. 33, Box B) are widespread in Galapagos; they show no differences in genetic structure across the archipelago (Santiago-Alarcon et al., 2010). Using primers described by Perkins and Schall (2002), Santiago-Alarcon et al. (2010) found several hemoproteid lineages, which are closely related to lineages of *H. multipigmentatus*. These lineages were found in 10 species of birds in the New World, i.e., the Zenaida dove (*Zenaida aurita*; Caribbean Islands), eared dove (Ecuador and Venezuela), Pacific dove (*Z. meloda*; Peru), Ecuadorian ground dove (*Columbina buckleyi*; Ecuador), croaking ground dove (*C. cruziana*; Ecuador), ruddy ground dove (*C. talpacoti*; Guatemala), rock dove (Ecuador), grey-headed dove (*Leptotila plumbeiceps*; Mexico), Inca dove (*Scardafella inca*; Guatemala), and ruddy quail-dove (*Geotrygon montana*; Ecuador). Further investigation of blood stages of the parasites is needed to prove whether any of them belong to *H. multipigmentatus*.

The Galapagos dove is endemic to Galapagos and is widespread in the archipelago (Santiago-Alarcon et al., 2006; Santiago-Alarcon and Parker, 2007); therefore, it serves as a convenient model organism in studies of ecology and evolution of parasitic diseases in geographically restricted, but highly mobile, hosts (Parker et al., 2006; Santiago-Alarcon et al., 2010). The present study shows that *H. multipigmentatus* is a highly prevalent and widespread hemoproteid of the Galapagos dove. Because the same, or closely related, lineages of *H. multipigmentatus* are present in several species of columbiform birds in the New World, this parasite certainly has a wide range of transmission, as is the case with some other species of avian hemoproteids (Bishop and Bennett, 1992; Valkiūnas, 2005; Bensch et al., 2009). To date, *H. multipigmentatus* and its lineages have been recorded in countries in the New World with warm climates. Recent genetic studies suggest that *H. multipigmentatus* is a relatively new arrival to the archipelago, probably from different continental dove populations (Santiago-Alarcon et al., 2010). Closely related lineages of *H. multipigmentatus* have been recorded in continental populations of the eared dove; this bird is widely distributed in South America and also has been recorded as a vagrant species in Galapagos (Curry and Stoleson, 1988). It is possible that vagrant eared doves could have naturally introduced *H. multipigmentatus* into the Galapagos Islands. Rock doves were also repeatedly introduced to the archipelago, and so might also be a source of infection for the Galapagos dove. However, lineages of *H. multipigmentatus* have not been recorded in the rock doves in Galapagos (P. Parker, unpubl. obs.) and have not been documented in continental populations of this bird. Thus, the rock dove, which was eradicated from the archipelago in 2002, seems a less probable source of infection for the Galapagos dove. Additional studies of hemoproteids in continental populations of columbiform birds are needed to understand the origin of *H. multipigmentatus* in Galapagos.

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