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Hippoboscid-transmitted *Haemoproteus* parasites (Haemosporida) infect Galapagos Pelecaniform birds: Evidence from molecular and morphological studies, with a description of *Haemoproteus iwa* ☆

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ABSTRACT

Haemosporidian parasites are widely distributed and common parasites of birds, and the application of molecular techniques has revealed remarkable diversity among their lineages. Four haemosporidian genera infect avian hosts (*Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Fallisia*), and *Haemoproteus* is split into two sub-genera based on morphological evidence and phylogenetic support for two divergent sister clades. One clade (*Haemoproteus* (*Parahaemoproteus*)) contains parasites developing in birds belonging to several different orders, except pigeons and doves (Columbiformes), while the other (*Haemoproteus* (*Haemoproteus*)) has previously been shown to only infect dove hosts. Here we provide molecular and morphological identification of *Haemoproteus* parasites from several seabird species that are closely related to those found in dove hosts. We also document a deeply divergent clade with two haemosporidian lineages recovered primarily from frigatebirds (Fregatidae, Pelecaniformes) that is sister to the hippoboscid-(Hippoboscidae) transmitted dove parasites. One of the lineages in this new clade of parasites belongs to *Haemoproteus iwa* and is distributed in two species of frigatebird (*Fregata*) hosts from Hawaii, the Galapagos Islands, the eastern Pacific and throughout the Caribbean Basin. Haemosporidian parasites are often considered rare in seabirds due in part to the lack or low activity of some dipteran vectors (e.g., mosquitos, biting midges) in marine and coastal environments; however, we show that *H. iwa* is prevalent and is very likely vectored among frigatebirds by hippoboscid flies which are abundant on frigatebirds and other seabirds. This study supports the existence of two sister clades of avian *Haemoproteus* in accord with the subgeneric classification of avian hemoproteids. Description of *H. iwa* from Galapagos *Fregata minor* is given based on morphology of blood stages and segments of the mitochondrial cytochrome *b* gene, which can be used for identification. This study shows that hippoboscid flies warrant more attention as vectors of avian *Haemoproteus* spp., particularly in marine and coastal environments.

1. Introduction

Haemosporidian parasites are ecologically successful apicomplexans (protists) found in birds, reptiles and mammals from nearly all regions of the world aside from those close to the poles (Valkiūnas, 2005). Parasitologists have described numerous genera and subgenera within the order Haemosporida (phylum: Apicomplexa) containing several hundred named species and at least 500 mtDNA haplotypes (Bensch et al., 2009). These parasites are vector-borne and have been associated with transmission by species from at least seven families of Diptera (Levine, 1988). Avian haemosporidians include parasites from four genera: *Plasmodium*, which is typically vectored by mosquitoes (Culicidae); *Haemoproteus*, which is primarily transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae); *Leucocytozoon*, which is

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vectored primarily by blackflies (Simuliidae) (only *Leucocytozoon caulleryi* is known to be transmitted by biting midges); and *Fallisia*, whose vectors are still unclear (Valkiūnas, 2005). The application of molecular techniques to the study of haemosporidian parasites has revealed a remarkable amount of genetic diversity, suggesting the existence of many undescribed (in many cases probably cryptic) species that share convergent morphological traits with described taxa (Ricklefs and Fallon, 2002; Bensch et al., 2004; Križanauskienė et al., 2006).

A recent reconstruction of the phylogeny of haemosporidian parasites using sequence data from four genes from each of the parasites' three genomes (nuclear, mitochondrial, plastid) and spanning lizard, bird and mammal parasites (Martinsen et al., 2008) suggests two non-sister clades within avian *Haemoproteus*. One clade (represented by three sequences of *Haemoproteus columbae* in Martinsen et al. (2008)) consists of parasites belonging to *Haemoproteus* (*Haemoproteus*) found in doves and is sister to all other ingroup taxa while other avian haemoproteids, (*Haemoproteus* (*Parahaemoproteus*)) found in non-columbiform hosts, form a clade that is sister to *Plasmodium* in mammals, birds and lizards (Martinsen et al., 2008). Santiago-Alarcon et al. (2010) documented additional diversity in *Haemoproteus* (subgenus *Haemoproteus*).

Haemosporidian parasites are common in continental regions but some species also occur on islands. Island populations of potential hosts are often more susceptible to introduced pathogens, as they have historically been exposed to fewer pathogens than mainland populations (e.g., Fromont et al., 2001). The Galapagos Islands are located on the equator approximately 1000 km west of continental Ecuador and have only been inhabited by humans for 200 years. Much of their biodiversity remains intact, with only 5% species loss (Gibbs et al., 1999). The isolation and high degree of endemism in the biota raise concerns about the introduction of diseases. Introduced pathogens, including avian pox (*Avipoxvirus*) and avian malaria (*Plasmodium relictum*) are a likely cause of major population declines and extinctions (Smith et al., 2006) (see effects of *P. relictum* on the Hawaiian avifauna; van Riper et al., 1986, 2002; Atkinson et al., 2000). Ongoing disease monitoring is an essential part of conservation efforts in Galapagos (Parker et al., 2006). A health survey of four Galapagos seabirds was conducted on the island of Genovesa in 2004 to establish species-specific baseline health parameters for future recognition of health-related threats to the endemic populations (Padilla et al., 2006). The survey discovered *Haemoproteus* sp. blood parasites infecting three of the four seabird species sampled (Great Frigatebird *Fregata minor*, Red-footed Booby *Sula sula* and Swallow-tailed Gull *Creagrus furcatus*). Parasite prevalence, estimated through microscopic examination of blood smears, ranged from 9% to 29% in the different bird species (Padilla et al., 2006). Blood parasites are considered rare in seabirds (e.g., Jovani et al., 2001), which might be related to competent immune defenses made possible by their long embryonic development periods (Ricklefs, 1992) or the lower abundance and/or low activity of some dipteran vectors (e.g., mosquitos, biting midges) in marine environments due to windy conditions and high salinity (Piersma, 1997; Mendes et al., 2005). Only a handful of published studies document *Haemoproteus* spp. in seabirds, three of which report *Haemoproteus* parasites in frigatebirds: Great Frigatebirds in Hawaii (Work and Rameyer, 1996), Christmas Island Frigatebirds (*Fregata andrewsi*) (Quillfeldt et al., 2010) and Magnificent Frigatebirds (*Fregata magnificens*) in Mexico (Madsen et al., 2007a). In Galapagos, haemosporidian parasites have previously been identified in the Galapagos Dove (*Zenaida galapagoensis*), which has high prevalence and intensity infections and is known to move readily throughout the archipelago (Padilla et al., 2004; Santiago-Alarcon et al., 2006, 2008). Recently, a *Plasmodium* sp. parasite has been identified in Galapagos Penguins (*Spheniscus mendiculus*), which could potentially have negative consequences for the small and vulnerable penguin population (Levin et al., 2009).

Here we present a phylogeny of the blood parasites found in Galapagos birds, which reveals a new clade of *Haemoproteus* parasites found primarily in frigatebirds. The lineage in Galapagos frigatebirds was identified as *Haemoproteus iwa*. Because the original description of this parasite from Hawaiian birds (Work and Rameyer, 1996) is incomplete (there is no information about microgametocytes and only one macrogametocyte was illustrated), we provide a morphological description of blood stages of *H. iwa* from its type avian host *F. minor* in Galapagos. These samples are the same lineage as recorded in Hawaii (the type locality of *H. iwa*). In addition, we provide molecular evidence potentially identifying the vector of *H. iwa*. Using sequences that include those from known morphospecies of described haemosporidian parasites (e.g., Valkiūnas et al., 2007, 2008a, 2010), we are able to understand the placement of this new parasite clade relative to other known lineages (including other Galapagos lineages).

2. Materials and methods

2.1. Sample collection

Samples from Galapagos birds were collected between 2001 and 2010 on numerous field expeditions. Seabirds were captured by hand on the nest or near nesting sites. A blood sample was collected from the brachial vein and stored in lysis buffer. Hippoboscid flies were collected directly from birds while sampling. Flies were stored in 95% ethanol in the field and later at 4 °C in the laboratory until DNA extraction. Blood films collected in 2010 were air-dried within 5–10 s after their preparation. They were fixed in absolute methanol in the field and then stained with Giemsa in the laboratory. Blood samples of Magnificent Frigatebirds from Pacific Panama, Belize and the Cayman Islands were collected during the nesting seasons of 2007 and 2008. All samples were from chicks or adults tending active nests. Blood samples from Hawaiian Great Frigatebirds (both breeding adults and juveniles) were collected during the breeding season of 1999 from birds nesting or roosting on Tern Island.

2.2. Molecular screening

DNA was extracted from blood using a standard phenol–chloroform extraction protocol (Sambrook et al., 1989) and PCR was used to amplify regions of the parasite mitochondrial *cytochrome b* gene (mtDNA, *cyt b*). Positive and negative controls were always used and, in most cases, any individual sample that amplified was reamplified to confirm a true positive. Primers used to amplify and sequence parasite *cyt b* from birds tested in the University of Missouri – St. Louis, USA, laboratory included an initial outer reaction (HAEMNF and HAEMNR2) followed by an internal re-amplification (HAEMF and HAEMR2) (Waldenström et al., 2004). Reaction conditions for both sets of primers followed Waldenström et al. (2004). PCRs were performed using Takara Ex taq polymerase and accompanying reagents (Takara Bio Inc., Japan). One microlitre of stock DNA was used in the initial reaction and 0.5 μL of amplicon from the initial reaction was used as a template for the internal re-amplification reaction. PCR products were cleaned using Qiagen PCR Purification kits (QIAGEN) or using Exonuclease I and Antarctic Phosphatase (#M0289S and #M0293S, respectively, New England Bio Labs, Inc.). Four hundred and ninety eight base pairs of double-stranded DNA sequence were obtained using an Applied Biosystems 3100 DNA Analyzer at the University of Missouri – St. Louis with BigDye Terminator v3.1 Cycle Sequencing chemistry. The protocol used to amplify and sequence parasite DNA from

Galapagos seabirds tested at the University of Leeds, UK, also followed Waldenström et al. (2004), but used an annealing temperature of 52 °C in the internal reaction. For the University of Leeds samples, either Biotaq (Bioline, USA) or Flexi Go Taq (Promega, USA) DNA polymerase was used in these reactions. Samples were sequenced using an Applied Biosystems 3730 DNA Analyzer at the Medical School at the University of Sheffield, UK, with BigDye Terminator v3.1 Cycle Sequencing chemistry. Sequences were obtained from haemosporidian parasites from 64 *F. minor* (eight from Hawaii and 56 from Galapagos), 18 *F. magnificens* (10 from Galapagos, two from Pacific Panama, two from Belize, four from the Cayman Islands), two *S. mendiculus* (Galapagos), seven *Z. galapagoensis* (Galapagos), five *Sula nebouxii* (Galapagos), two *C. furcatus* (Galapagos) and five *Olfersia* spp. hippoboscid flies (Galapagos). Twenty-nine additional frigatebird parasites (26 *F. minor* and three *F. magnificens*) were also sequenced using the *caseinolytic protease* gene (*ClpC*) following Martinsen et al. (2008).

In the laboratory, thoraces of 20 hippoboscid flies were carefully separated from heads and abdomens. Each thorax was used individually for DNA extraction using a Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, USA). The standard protocol was followed, but DNA was eluted in half as much 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 undigested parasite-infected blood cells that might have persisted in the vector digestive system as remnants of a blood meal, thoraces of the seven insects that tested positive for *Haemoproteus* were tested for the bird mitochondrial *cyt b* gene with primers and protocols used in Ngo and Kramer (2003). Frigatebird mtDNA was used as a positive control to identify and compare bird DNA amplified from insect thoraces. New sequences were deposited in GenBank (accession numbers: JF833042–JF833066).

2.3. Phylogenetic analyses

Cyt b sequences were edited in Seqman 4.0 [DNASTAR, USA], added to a larger dataset containing additional *cyt b* sequence data obtained from GenBank (Supplementary Table S1), and aligned using BioEdit (Version 7.0.9.0; Hall, 1999). The best-fit model of DNA evolution was determined using jMODELTEST (Version 0.1.1) (Guindon and Gascuel, 2003; Posada, 2008). The GTR+I+Γ model of nucleotide substitution was used to reconstruct a maximum likelihood phylogeny and a maximum likelihood bootstrap analysis (500 pseudoreplicates) (Jobb, 2009: Treefinder http://www.treefinder.de). Bayesian posterior probabilities were obtained from 10 million trees using the programme BEAST (Drummond and Rambaut, 2007). BEAST initiates a pre-burn-in to stabilise likelihood values, after which it begins sampling. The likelihood stationarity of sampled trees was determined graphically using TRACER. Parameters in BEAST allow for mutation rate heterogeneity among branches of the phylogeny, reducing bias due to disproportionately long branches (relaxed clock: uncorrelated lognormal). Lineage birth was modeled using a Yule prior. Sequence divergence between the different lineages was calculated in MEGA 3.1 (Kumar et al., 2004).

2.4. Microscopic examination

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 et al. (2008b). Intensity of infection was estimated as a percentage by counting 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 haemosporidian parasites in the type voucher material of *H. iwa*, the entire blood films were examined microscopically at low magnification.

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, prepare illustrations and to take measurements. The morphometric features studied (Table 1) are those defined by Valkiūnas (2005). Morphology of *H. iwa* from Galapagos material was compared with the parahapantotypes of *H. iwa* (Accession Nos. G212808, G212809 and G212810 in the Queensland Museum, Brisbane, Queensland, Australia). Student's *t*-test for independent samples was used to determine statistical significance between mean linear measurements. A *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Phylogenetic analyses

Our phylogenetic analyses suggest two major groups, *Plasmodium* and *Haemoproteus*, with *Haemoproteus* further split into two divergent sister clades *Haemoproteus* (*Haemoproteus*) and *Haemoproteus* (*Parahaemoproteus*) (Fig. 1). Clade A contains parasites found primarily in pigeons and doves (*Haemoproteus* (*Haemoproteus*)), which is sister to a new clade of parasites found primarily in frigatebird hosts (clade B) (Fig. 1). Lineages of haemosporidian parasites from both *Plasmodium* and *Haemoproteus* were found in Galapagos birds. Five sequences from Blue-footed Boobies (*S. nebouxii*) clustered with *Haemoproteus* (*Parahaemoproteus*) and *Plasmodium* parasites were found in Galapagos penguins (*S. mendiculus*). The Blue-footed Booby parasite sequences were generated using the same primers as those used to amplify other Galapagos seabird parasites, with no indication of mixed infections (e.g., no double peaks in the chromatogram). Most of the recorded sequences cluster with *Haemoproteus* (*Haemoproteus*) and are split between two major clades (labelled A and B). Clade A contains parasites from Rock Pigeons (*Columba livia* infected with *Haemoproteus columbae*) (non-Galapagos sequences that have been used in other studies to represent the *H.* (*Haemoproteus*) sub-genus), Galapagos Doves (numerous lineages of *Haemoproteus multipigmentatus*) and unidentified *Haemoproteus* lineages from three seabird species (Nazca Booby (*Sula granti*), Magnificent Frigatebird and Swallow-tailed Gull (*C. furcatus*) (clade A). Hippoboscid flies and frigatebirds (*F. minor* and *F. magnificens*) from Galapagos, Hawaii (*F. minor*), Caribbean (Belize and Cayman Islands) (*F. magnificens*) and Pacific coasts of Panama (*F. magnificens*) as well as one Swallow-Tailed Gull (*C. furcatus*, also from Galapagos) were infected with *Haemoproteus* parasites that formed a well-supported and hitherto undescribed clade (clade B) which is sister to clade A (Fig. 1). Average pairwise sequence divergence between clade A and clade B is 8%. There is no genetic variation among all sequences from frigatebird parasites (clade B); as mentioned above, one Magnificent Frigatebird parasite sequence clustered with the clade A containing mostly dove parasites, while all others ($n = 82$) were identical for the *cyt b* fragment sequenced and encountered in Pacific and Caribbean *F. minor* ($n = 8$ from Hawaii and $n = 56$ from Galapagos) and *F. magnificens* ($n = 10$, 2, 2, 4 from Galapagos, Pacific Panama, Belize and Cayman Islands, respectively). To avoid redundancy, only one to two from each species/location of these sequences are shown in Fig. 1. Parasites from all Galapagos

Table 1
Morphometry of host cells and fully-grown gametocytes of *Haemoproteus iwa* from the great frigatebird *Fregata minor*.

Feature	Measurements (μm)[a]
Uninfected erythrocyte	
Length	14.3–16.6 (15.2 ± 0.5)
Width	6.6–8.4 (7.6 ± 0.5)
Area	84.5–108.0 (95.1 ± 6.6)
Uninfected erythrocyte nucleus	
Length	6.1–8.2 (7.0 ± 0.5)
Width	2.1–3.7 (2.6 ± 0.4)
Area	10.7–19.4 (14.5 ± 2.3)
Macrogametocyte	
Infected erythrocyte	
Length	13.2–17.7 (16.2 ± 1.2)
Width	6.9–10.2 (8.3 ± 0.9)
Area	78.8–123.5 (108.6 ± 9.8)
Infected erythrocyte nucleus	
Length	5.7–7.3 (6.8 ± 0.4)
Width	2.1–2.7 (2.3 ± 0.1)
Area	9.7–15.6 (13.5 ± 1.5)
Gametocyte	
Length	15.5–19.6 (17.9 ± 1.1)
Width	3.3–5.7 (4.3 ± 0.6)
Area	60.1–82.0 (74.0 ± 5.2)
Gametocyte nucleus	
Length	2.6–4.4 (3.5 ± 0.5)
Width	1.8–3.4 (2.4 ± 0.4)
Area	3.9–8.0 (6.2 ± 1.2)
Number of pigment granules	49–67 (57.4 ± 5.1)
NDR[b]	0.2–0.5 (0.4 ± 0.1)
Microgametocyte	
Infected erythrocyte	
Length	13.0–18.0 (15.3 ± 1.5)
Width	7.1–11.0 (8.5 ± 0.9)
Area	87.3–133.7 (105.6 ± 12.5)
Infected erythrocyte nucleus	
Length	6.1–8.2 (7.2 ± 0.6)
Width	1.9–2.9 (2.3 ± 0.3)
Area	11.6–16.2 (14.1 ± 1.1)
Gametocyte	
Length	14.6–20.9 (17.3 ± 1.6)
Width	3.0–4.2 (3.5 ± 0.3)
Area	40.5–74.7 (53.2 ± 8.9)
Gametocyte nucleus[c]	
Length	–
Width	–
Area	–
Pigment granules	25–40 (31.7 ± 3.4)
NDR	0.5–0.9 (0.7 ± 0.1)

[a] All measurements ($n = 21$) are given in micrometres. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and S.D.
[b] NDR is nucleus displacement ration according to Bennett and Campbell (1972).
[c] Due to a markedly diffuse nucleus, its measurement is difficult (see description of the parasite, Section 3.2).

frigatebirds were morphologically identical; they belong to *H. iwa* (see description below).

We obtained 20 *Haemoproteus ClpC* sequences from Galapagos frigatebirds, seven from Hawaiian frigatebirds and two from Caribbean/Pacific Panamanian frigatebirds and found that the results were consistent with the *cyt b* gene; there was no variation in clade B containing primarily frigatebird parasites, which form a well supported clade as with *cyt b*.

Seven parasite DNA sequences were recovered from thoraces of hippoboscid flies collected from Great Frigatebirds and they were identical to the lineage found in clade B (Fig. 1). It is unlikely that the detected parasite DNA was from gametocytes remaining in blood meals because no bird DNA could be amplified from the thoraces.

3.2. Description of Haemoproteus (Haemoproteus) iwa Work and Rameyer (1996) from Fregata minor in the Galapagos Islands

Young gametocytes. Earliest forms were not seen in voucher material.

Macrogametocytes (Fig. 2A–H). Extend along nuclei of erythrocytes and displace the nuclei laterally from early stages of their development (Fig. 2A–C), which is a characteristic feature of parasite development. Elongate broadly-halteridial bodies with even or slightly irregular outline, but more frequently the former; amoeboid forms not seen. Cytoplasm blue, homogeneous in appearance, often possesses prominent vacuoles of variable size (Fig. 2B–E, H); volutin granules not seen. Both growing (Fig. 2A and B) and fully-grown gametocytes (Fig. 2E and F) appressed to erythrocyte envelope but do not touch erythrocyte nuclei. A few fully-grown gametocytes were seen in association with erythrocyte nuclei; if present, such association is superficial and often disconnected at 1 or several points (Fig. 2G and H). Parasite nucleus markedly variable in form, frequently irregular in shape, submedial or medial in position (Fig. 2A–H). Nucleolus frequently seen (Fig. 2C); occasionally, 2 nucleolus-like clumps of chromatin were visible (Fig. 2H). Pigment granules of small (<0.5 μm) and medium (0.5–1 μm) size, roundish, irregular or oval in form, black, very numerous (Table 1), randomly scattered throughout cytoplasm. Size and number of pigment granules increase as parasite matures (compare Fig. 2A–H, E–H). Fully-grown gametocytes only slightly enclose erythrocyte nuclei with their ends, filling erythrocytes up to their poles (Fig. 2E–H); they markedly displace nuclei of erythrocytes laterally (Fig. 2F and G), frequently to envelope of erythrocytes (Fig. 2H). Infected erythrocytes are hypertrophied and their nuclei atrophied in length, width and area compared with uninfected erythrocytes (Table 1, $P < 0.01$ for all of these characters).

Microgametocytes (Fig. 2I–L). General configuration as for macrogametocytes with usual hemosporidian sexually dimorphic characters. Gametocytes do not touch erythrocyte nuclei; this feature is more evident in fully-grown microgametocytes than in macrogametocytes (compare Fig. 2F–H, J–L). Outline more irregular and fewer vacuoles than in macrogametocytes (compare Fig. 2A–H, I–L); amoeboid forms present (Fig. 2L). Cytoplasm is of reddish shade, partly due to markedly diffuse parasite nuclei, boundaries of which are unclear, making nuclei difficult to measure. Number of pigment granules is approximately one-half that in macrogametocytes (Table 1, $P < 0.001$). Pigment granules lighter in colour (usually brown) than in macrogametocytes; the majority of granules tend to group and to gather close to ends of gametocytes, but individual granules can be seen anywhere in the cytoplasm (Fig. 2K and L). Fully-grown microgametocytes are more slender in form and displace host nuclei less than macrogametocytes (Table 1, compare Fig. 2G, H, K, L).

3.2.1. Taxonomic summary

Avian hosts. Fregata minor, F. magnificens (Pelecaniformes).

Distribution. H. iwa and its *cyt b* lineages were recorded on Hawaii, Galapagos, Eastern Pacific and Caribbean coast/islands; it is probably widespread in the range of distribution of frigatebirds.

Voucher specimens. Blood films (intensity of parasitemia is approximately 0.01%, *Fregata minor*, North Seymour, Galapagos, 00°23′38″S, 90°17′32″W, lineage FminGal1, collected by I. Levin, 6 July 2010) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania (Accession Nos. 47740 NS, 47741 NS), in the US National Parasite Collection, Beltsville, MD, USA (USNPC 104268, 104269), and in the Queensland Museum, Brisbane, Australia (G465451, G465452).

Additional material. Thirty-two slides (Accession Nos. 47744–47775 NS) where intensity of parasitemia is <0.001%, other data as for voucher specimens, are deposited in the Institute of Ecology,

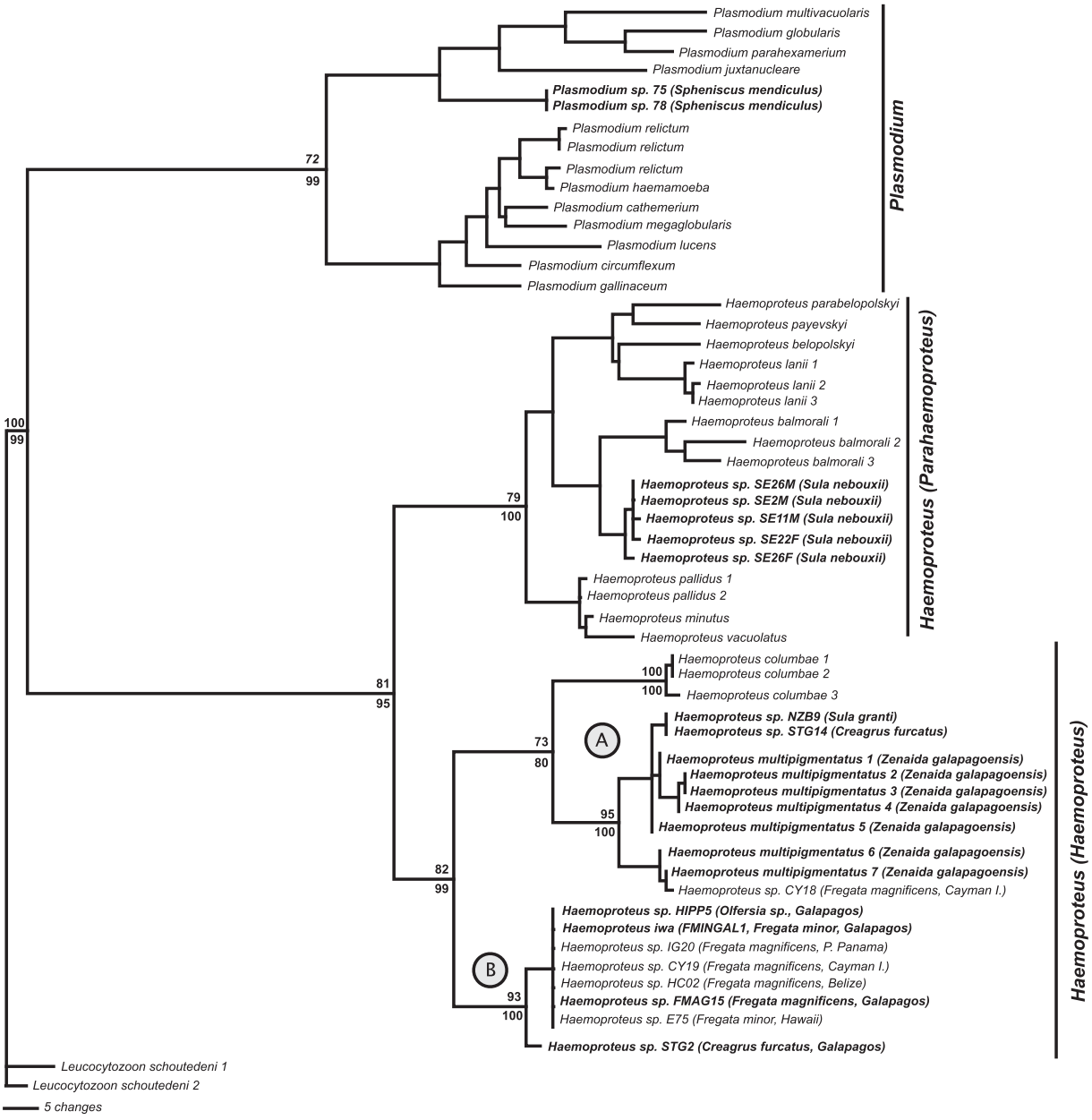

Fig. 1. Maximum likelihood (ML) phylogenetic hypothesis of haemosporidian parasites based on 524 bp of the mitochondrial *cyt b* gene. ML bootstrap values appear above the nodes and Bayesian posterior probabilities appear below the nodes. Clades A and B belong to the subgenus *Haemoproteus*; sequences in clade A are mostly parasite lineages restricted to Columbiformes, whereas clade B sequences are parasite lineages restricted to frigatebirds (with one exception of one lineage found in a Swallow-Tailed Gull). For previously unpublished sequences, host species appear in parentheses; sequences from Galapagos are in bold. Parasite lineages are detailed in Supplementary Table S1 and listed in the order in which they appear in the phylogeny.

Nature Research Centre, Vilnius, Lithuania. Duplicates of these slides are also available at the University of Missouri – St. Louis, USA.

DNA sequences. Mitochondrial *cyt b* lineage FminGal1 (GenBank™ Accession No. JF833050) can be used for molecular identification of *H. iwa*.

Vector. Olfersia spinifera (Diptera, Hippoboscidae) is a probable vector in Galapagos.

Prevalence. In Galapagos, the overall prevalence of infection in Great Frigatebirds based on PCR detection was 113 of 204 (55.4%).

3.2.2. Remarks

Haemoproteus iwa can be readily distinguished from other avian hemoproteids due to the large number of pigment granules in its macrogametocytes (Table 1), which is approached only by *H. multipigmentatus* in the columbiform, *Z. galapagoensis* (see Valkiūnas et al., 2010); the former species nonetheless produces more pigment granules (average number of the granules in macrogametocytes of these parasites is 57 and 43, respectively, $P < 0.001$). Interestingly, both of these parasites produce more pigment granules in macrogametocytes than any other described species of avian hemoproteid, and are therefore similar from this point of view. In *H. iwa* (i) mature gametocytes are often not appressed to erythrocyte nuclei, which is particularly evident in microgametocytes (see Fig. 2J–L), (ii) macrogametocytes often possess prominent vacuoles (Fig. 2A–E, HA–E, H), and (iii) the number of pigment granules in macrogametocytes is at least twice that in microgametocytes (Table 1). None of these readily distinguishable features of *H. iwa* are characteristics of *H. multipigmentatus*.

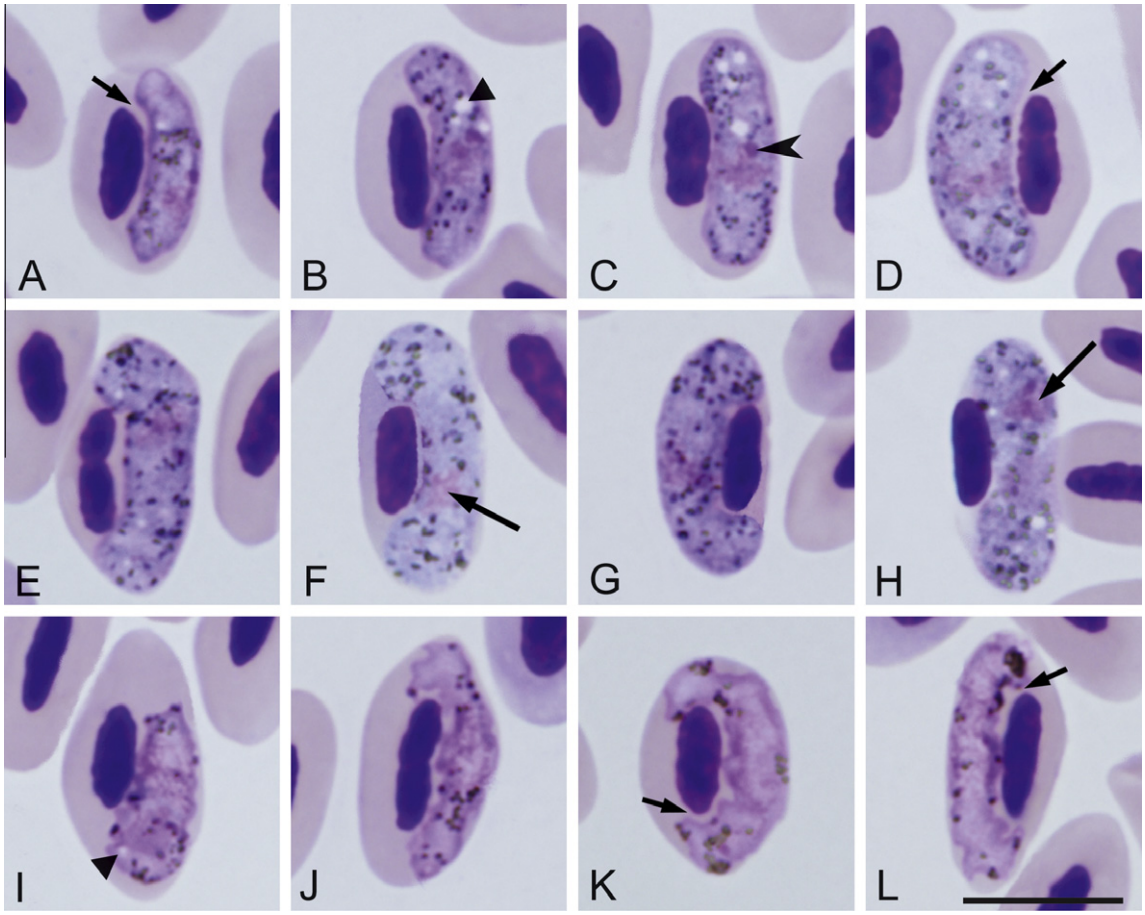

Fig. 2. *Haemoproteus* (*Haemoproteus*) *iwa* from the blood of the Great Frigatebird *Fregata minor* in Galapagos. (A–H) Macrogametocytes, (I–L) microgametocytes. Long arrows – nuclei of parasites, short arrows – unfilled spaces among gametocytes and nuclei of infected erythrocytes. Large arrow head – nucleolus. Small arrow heads – vacuoles. Giemsa-stained thin blood films. Bar = 10 μm.

A full range of blood stages of *H. iwa* (except earliest gametocytes) is published for the first time (Fig. 2A–L). Microgametocytes were not reported in the original description of *H. iwa,* probably due to extremely light infection (Work and Rameyer, 1996), but they are present in voucher material of this parasite from Galapagos (Fig. 2I–L). Macrogametocytes are more numerous than microgametocytes; the ratio in the voucher material is 2.5:1.

4. Discussion

According to current knowledge, parasites from the subgenus *Haemoproteus* (currently only seven species defined mainly by morphological and life history traits) infect birds only from the order Columbiformes (Valkiūnas et al., 2010). Thus, this is the first time that close phylogenetic relatives of parasites belonging to the subgenus *Haemoproteus* have been found and documented both by PCR and microscopy in non-columbiform hosts. This parasite is *H. iwa*, which is the first representative of the subgenus *Haemoproteus* infecting non-columbiform birds. *Haemoproteus iwa* was originally described from the Great Frigatebird in Hawaii (Work and Rameyer, 1996); the original description is incomplete (microgametocytes were not described) and is based on extremely light infections (only four gametocytes of the parasite were seen in this species' parahapantotype material after a 4 h examination, G. Valkiūnas personal observation). During this study, we detected the same lineages of *H. iwa* in Great Frigatebirds in both Hawaii and Galapagos. Because (i) parasitemia was relatively high, (ii) the main morphological features of Hawaiian and Galapagos parasites are similar, and (iii) the same *cyt b* haplotype was present in Great Frigatebirds in Hawaii and Galapagos, our material provided an opportunity to prepare a morphological re-description of *H. iwa* that is important for future taxonomic and ecological studies.

While some of the seabirds (Nazca Booby NZB9, Magnificent Frigatebird CY18, Swallow-Tailed Gull STG14; see Fig. 1) appear to be infected by parasite lineages very similar in DNA sequence to *H. multipigmentatus* infecting the Galapagos doves (clade A), the majority of the frigatebirds (and one Swallow-Tailed Gull) are infected with parasites that form their own, well-supported sister clade within the subgenus *Haemoproteus* (clade B). Diversity reported in clade B has never been described, perhaps due to under-representation in sampling for molecular studies of parasites infecting marine and coastal birds. The detection of what is likely *H. multipigmentatus* in the occasional seabird (*S. granti*, *C. furcatus*, clade A) could represent sporozoites injected into the bloodstream from a bite by *Microlynchia galapagoensis*, the Hippoboscid fly normally parasitising doves (Valkiūnas et al., 2010). Doves were seen near seabird colonies (I. Levin, personal observation) and PCR protocols can amplify sporozoites from the peripheral blood of birds (Valkiūnas et al., 2009). It remains unclear whether *H. multipigmentatus* can complete development in seabirds to gametocyte stage. Thus, the detection of parasite DNA in the blood does not provide evidence that the parasite can complete its lifecycle in these seabird species. This warrants further investigation and exemplifies the need for studies that include both molecular and microscopical approaches.

Clade B does not appear to be unique to the Galapagos, as DNA sequences from parasites infecting Hawaiian, Pacific Panamanian and Caribbean Magnificent Frigatebirds have the same sequence as parasites in Galapagos frigatebirds. Thus, *H. iwa* has a wide range of distribution and infects different species of frigatebirds. This is similar to the results found for *H. multipigmentatus* infecting Columbiformes, where this parasite is not endemic to the Galapagos but is widely distributed across the American continent (Santiago-Alarcon et al., 2010). Based on molecular evidence (Fig. 1, clade B), it is possible that *H. iwa* also completes development in the gull, *C. furcatus*, but detection of blood stages is needed for confirmation.

In order to assess the lack of sequence diversity in *cyt b* (one parasite haplotype for all clade B frigatebird parasites), we amplified and sequenced a portion of the parasite's plastid genome, *ClpC*, for a subset of samples. Santiago-Alarcon et al. (2010) found that *ClpC* was more variable at the tips of the parasite phylogeny; thus, it provided a better resolution of the relationships among haplotypes of *H.* (*Haemoproteus*) spp. in doves when *cyt b* did not (see also Outlaw and Ricklefs, 2009). We obtained 29 *Haemoproteus ClpC* sequences from Galapagos, Hawaiian, Panamanian and Caribbean frigatebirds and found that the results were consistent with the *cyt b* gene; we observed no variation in clade B sequences containing primarily parasites of frigatebirds. In contrast, Great Frigatebirds from Hawaii and Galapagos show strong genetic differentiation at mitochondrial and nuclear loci (Hailer et al., unpublished data). Furthermore, within Magnificent Frigatebirds, the Galapagos population has apparently been isolated from conspecific populations in the Pacific and Atlantic since the Pleistocene era (Hailer et al., 2010). In the light of these findings, sharing of the same *H. iwa* lineage among frigatebirds from diverse geographic locations reported here suggests either a very slow rate of sequence evolution in clade B, or transmission of the parasite among frigatebird populations in the absence of host gene flow.

A possible sequence divergence rate for haemosporidian *cyt b* has recently been estimated at 1.2% per million years for lineages infecting passerine birds (Ricklefs and Outlaw, 2010). Using this estimate (assuming the rate also applies to haemosporidians of non-passeriform birds) and colonisation times of Magnificent Frigatebirds to the Galapagos calculated by Hailer et al. (2010), we can estimate the probability that Galapagos lineages would not have diverged since the host colonised the archipelago. Based on a geometric mean colonisation time of 247,200 years before the present, the probability of no nucleotide changes in 524 bp of *cyt b* since colonisation is 0.21. For the 95% confidence limits of the frigatebird colonisation time (Hailer et al., 2010), we estimated the probability of no divergence to be 0.59 for the most recent colonisation estimate (82,800 years before present (YBP)) and 0.015 for the most ancient (647,400 YBP). Therefore, the absence of differentiation between this and source lineages of the frigatebird haemosporidian is not incompatible with arrival of the parasite with the colonising population of frigatebirds.

The well-supported clade of primarily frigatebird haemosporidian parasites, which is sister to clades of hippoboscid-transmitted *H. columbae* and *H. multipigmentatus*, indicates that subgeneric classification of haemoproteids remains valid and we cannot continue to consider *H.* (*Haemoproteus*) blood parasites to be columbiform-specific. Importantly, *H. iwa* haplotypes were present in thoraces of hippoboscid flies. A possibility for transmission of this parasite by hippoboscid flies was speculated by Work and Rameyer (1996) and Valkiūnas (2005, p. 861), but there has been no supporting evidence for this to date. Although several recent publications have reported blood parasites in non-passerines (e.g., Mendes et al., 2005; Krone et al., 2008; Ishak et al., 2008; Ortego et al., 2008; Outlaw and Ricklefs, 2009; Yohannas et al., 2009; Quillfeldt et al., 2010), none of these have identified parasites belonging to, or closely related to, the subgenus *Haemoproteus*.

Haemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of vectors and undergo markedly different sporogony, and therefore differ genetically and appear in different clades in phylogenetic trees (Martinsen et al., 2008; Santiago-Alarcon et al., 2010). Briefly, species of *H.* (*Haemoproteus*) are transmitted by flies belonging to the Hippoboscidae and are characterised by large oocysts (>20 μm in diameter) that possess numerous germinal centres, many sporozoites in mature oocysts (>500) and relatively short sporozoites (mean less than 10 μm) that are usually blunt at one end and pointed at the other (Baker, 1966; Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005). None of these characteristics are features of *H.* (*Parahaemoproteus*) spp. Development of *H. iwa* in hippoboscid flies remains undescribed.

Concerning *H. iwa*, a possibility for transmission by hippoboscid flies was speculated by Work and Rameyer (1996) and Valkiūnas (2005, p. 861). Here we provide molecular evidence that suggests that hippoboscid flies (*Olfersia* sp., probably *Olfersia spinifera* from reports of this fly parasitising frigatebirds) are the vectors for *H. iwa* among frigatebirds, based on identical parasite DNA sequences amplified from hippoboscid thoraces. These ectoparasitic flies are common on frigatebirds and related Pelecaniforms, even in the dry climates of Galapagos coastal habitat (I. Levin, personal observation). Because parasite DNA, but no bird DNA, was recovered from fly thoraces, it is likely that the sequences came from the sporozoites of *H. iwa*. The sporozoite is the only sporogonic stage in avian haemosporidians that is present in thoraces and salivary glands of the vectors, including hippoboscid flies (Baker, 1966; Valkiūnas, 2005). Biting midges have also been documented as vectors for *Haemoproteus* parasites; however, they have not been caught in traps near seabird colonies in Galapagos (J. Rabenold, personal communication). Biting midges typically require higher humidity and are therefore less likely to occur at these dry and windy coastal sites. Our molecular evidence and ecological observations provide strong support for *Olfersia* sp. hippoboscid flies as the vector for *H. iwa*, but detection of oocysts in the mid-gut and sporozoites in the salivary glands of the flies ideally followed by experimental infection of uninfected seabirds by sporozoites would be necessary for complete confirmation of the vector. Given that Galapagos frigatebird *H. iwa* parasites were identical at this region of *cyt b* to parasites from frigatebirds across the New World tropics – despite the genetic isolation of the Galapagos Magnificent Frigatebird – it is possible that the parasite is being moved between populations of frigatebirds during the non-breeding season via the transfer of the hippoboscid fly vectors at roosting sites where populations of frigatebirds might interact but apparently do not interbreed. Given this possibility, we confirmed the infection status of chicks and juvenile *F. minor* from Galapagos. Five of 20 chicks and 18 of 22 juveniles were infected with *H. iwa*, providing evidence that this lineage is locally transmitted in the Galapagos.

It is unclear whether hemoproteids pose a health threat to their Galapagos hosts. *Haemoproteus* parasites are typically considered benign by most veterinarians, but recent experimental evidence shows some fitness consequences for infected hosts in the wild (e.g., Merino et al., 2000; Marzal et al., 2005). It is important to note that some species of avian *Haemoproteus* cause severe pathology in birds (Cardona et al., 2002) and are sometimes lethal (Ferrell et al., 2007). Additionally, male Magnificent Frigatebirds infected with *H. iwa* tended to have lighter coloured red gular pouches (Madsen et al., 2007a), although there is not evidence for a role of gular pouch colour in mate choice in frigatebirds (Madsen et al., 2007b). Finally, Galapagos Great Frigatebirds infected with *Haemoproteus* spp. had significantly higher heterophil-to-lymphocyte concentration ratios than uninfected individuals, indicating that they were physiologically

stressed or actively fighting an infection (Padilla et al., 2006). Further studies are needed to understand the pathogenicity of *H. iwa*.

In conclusion, we have documented *H. iwa* and closely related lineages of haemosporidian parasites from Galapagos seabirds that are closely related to parasites that have previously only been found in dove and pigeon hosts. In addition, we have provided molecular evidence for a deeply divergent haemosporidian clade recovered primarily from frigatebirds that is sister to the dove and pigeon parasite clade. These parasites from frigatebirds show no genetic variation at *cyt b*, even across broad geographic scales. We provide evidence that *H. iwa* is likely vectored by the hippoboscid fly, *O. spinifera*, which is abundant on frigatebirds and other seabirds (Work and Rameyer, 1996; Quillfeldt et al., 2010). Characterising these parasites by placing them in a phylogenetic context with other previously described taxa is the first step in understanding their evolutionary history and their host breadth. Importantly, molecular evidence from this study shows that species of the Hippoboscidae are likely vectors not only of *H. iwa* but also of avian *Haemoproteus* spp. of other marine and coastal birds (Fig. 1 clades A and B). This finding indicates that hippoboscid flies warrant more attention as possible vectors of hemoproteids among not only columbiform birds, but also among non-columbiform birds, particularly in marine and coastal environments. Future studies should focus on the population level transmission dynamics of these haemosporidian parasites and explore the role of the vector in moving the parasites across large geographic distances as these parasite genetic data might suggest. The striking contrast between the genetically isolated Galapagos frigatebird host and the very widespread parasite is interesting and unexpected, and warrants future research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.03.014.

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