

INFECTION BY *HAEMOPROTEUS* PARASITES IN FOUR SPECIES OF FRIGATEBIRDS AND THE DESCRIPTION OF A NEW SPECIES OF *HAEMOPROTEUS* (HAEMOSPORIDA: HAEMOPROTEIDAE)

Santiago Merino, Janos Hennicke*†, Javier Martínez‡, Katrin Ludynia§||, Roxana Torres#, Thierry M. Work¶, Stedson Stroud**, Juan F. Masello§, and Petra Quillfeldt§

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, Madrid, Spain.
e-mail: santigom@mncn.csic.es

ABSTRACT: Among seabirds, the fregatids stand out with a high prevalence of blood parasites. Four of 5 species in this family have been found to be infected with *Haemoproteus*; however, complete species descriptions with molecular phylogeny are lacking. Seventy-five samples from 4 species of frigatebirds, i.e., *Fregata andrewsi*, *Fregata minor*, *Fregata magnificens*, and *Fregata aquila*, were screened for infections caused by species of *Haemoproteus*. Four different parasite haplotypes were found infecting frigatebirds based on the sequencing of a fragment of the *cytochrome b* gene. Two haplotypes belong to the subgenus *Parahaemoproteus*, and the other 2 correspond to haplotypes within the subgenus *Haemoproteus*. The more prevalent and cosmopolitan *Parahaemoproteus* haplotype (FregPHae1) was phylogenetically grouped with other *Haemoproteus* parasites infecting non-passerine birds, but it could not be detected from the single sample from *F. aquila*. The other *Parahaemoproteus* haplotype (FregPHae2) was not phylogenetically clustered with parasites infecting non-passerine birds, and it was sequenced from a single (1 each) *F. andrewsi* and *F. minor*. Blood smears from *F. andrewsi* infected only by FregPHae1 haplotype showed sufficient gametocytes to allow description of a new species, *Haemoproteus valkiünasi* sp. nov. In contrast to *Haemoproteus iwa*, the only previously known blood parasite infecting frigatebirds and described from *F. minor* from Galapagos Islands, parasites from *F. andrewsi* (1) are shorter with no contact of gametocyte with host cell membrane, (2) have fewer pigment granules, and (3) have wider microgametocytes, with a smaller host nuclear displacement. In contrast, patent single infections corresponding to the cosmopolitan haplotype of the subgenus *Haemoproteus* (FregHae1) were also found in samples from 1 *F. andrewsi*, 1 *F. minor*, and 1 *F. aquila*. In all these cases, the number of microgametocytes was very low, resembling *H. iwa*, which lacks microgametocytes in the original description. Macrogametocytes of haplotype FregHae1 in *F. andrewsi* differ significantly from all the characteristics measured from *H. valkiünasi*. In addition, it also differs from all characteristics of *H. iwa* despite being genetically identical in the analyzed fragment.

Seabirds have often been reported to be free from blood parasites, even in the presence of potential vectors. For example, a recent genetic screening study for infections caused by blood parasites (Quillfeldt et al., 2010) included 5 species of tropical seabirds breeding at Christmas Island in the Indian Ocean. No haemosporidian parasites were found in 4 species (Abbott's booby *Papasula abbotti* [12 individuals], red-footed booby *Sula sula rubripes* [12 individuals], brown booby *Sula leucogaster plotus* [12 individuals], and red-tailed tropicbird *Phaethon rubricauda* [12 individuals]), reflecting the overall scarceness in seabirds. However, 5 of 9 Christmas Island frigatebirds (*Fregata andrewsi*) were found to be infected with *Haemoproteus* sp. (Quillfeldt et al., 2010), and so were individuals representing *Fregata minor* from Hawaii (Work and Rameyer, 1996), *Fregata magnificens* from México (Madsen et al., 2007), *Fregata minor* and *Fregata ariel* from Aldabra Atoll, Indian Ocean (Lowery, 1971), and *F. minor*

from Genovesa, Galapagos Islands (Padilla et al., 2006). Therefore, frigatebirds are unique among seabirds in that they offer opportunities to evaluate blood parasites using morphological and genetic methods.

The 5 extant species of frigatebirds are closely related and belong to a single genus, *Fregata* (Fregatidae: Pelecaniformes). These are large birds, with wing spans ranging from 175 to 244 cm, but they weigh only from 600 to 1,600 g. Females are about 25% heavier than males. Frigatebirds are circumtropical pelagic piscivores, with a small amount of their diet obtained by kleptoparasitism (Osorno et al., 1992). They are essentially sedentary, spending most of the year in the vicinity of their colony, usually located on remote islands where nests are constructed in trees or on bare dry ground, and often close to other marine birds (Orta, 1992). A single egg is laid every 1–2 yr, and the duration of parental care in frigatebirds is one of the longest of any bird (between 150 and 428 days for great frigatebirds), which allows a long time for potential transmission of diseases between parents and offspring. However, young birds and non-breeding individuals disperse over vast areas, as do foraging adults.

The great frigatebird *Fregata minor* and the magnificent frigatebird *Fregata magnificens* both have very large distribution ranges, with several subspecies in different tropical oceans, and over 200,000 mature individuals per species (Bird Life International, 2011). In contrast, the critically endangered Christmas Island frigatebird (*Fregata andrewsi*) is an endemic species breeding on Christmas Island in the Indian Ocean (Orta, 1992; IUCN, 2010). It is the rarest of the 5 extant species of frigatebirds (2,400–4,800 mature individuals), and its distribution is restricted to the Indo-Malayan Archipelago encompassing southeastern Asia to Indochina and northern Australia. Population declines are attributed to habitat loss due to mining activities on Christmas Island, overfishing, hunting, and increased nestling

Received 17 December 2010; revised 24 March 2011, 28 September 2011; accepted 12 October 2011.

* Biozentrum Grindel, Universität Hamburg, Martin Luther King Platz 3, 20146 Hamburg, Germany.

† Centre d'Etudes Biologiques de Chizé, Centre National de la Recherche Scientifique, 79360 Villiers en Bois, France.

‡ Departamento de Parasitología, Universidad de Alcalá, Alcalá de Henares, Spain.

§ Max-Planck-Institut für Ornithologie, Vogelwarte Radolfzell, Schlos-sallee 2, 78315 Radolfzell, Germany.

|| Animal Demography Unit, Department of Zoology, University of Cape Town, Rondebosch 7701, Cape Town, South Africa.

Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, México City, D.F., México.

¶ U.S. Geological Survey, National Wildlife Health Center–Honolulu Field Station, P.O. Box 50167, 300 Ala Moana Boulevard, Room 5-231, Honolulu, Hawaii 96850.

** Ascension Island Government, Georgetown, Ascension Island, ASCN, 1ZZ.

DOI: 10.1645/GE-2415.1

mortality due to the introduced yellow crazy ant *Anoplolepis gracilipes* (Bird Life International, 2011; J. Hennicke, pers. obs.).

The Ascension frigatebird, *Fregata Aquila*, is also vulnerable (IUCN, 2010); it is represented by an estimated 17,000–20,200 individuals (Bird Life International, 2011), and breeds only on Boatswain Bird Island off the northeast coast of Ascension Island in the tropical Atlantic. It was exterminated from Ascension by introduced predators and humans. However, recent counts amounted to less than 900 birds present at the only breeding colony and, therefore, suggest that a revision of the population estimate is needed. As in other frigatebirds, little is known about its movement, although it does forage off western Africa.

Presently, the only species of *Haemoproteus* documented from pelecaniiforms is *Haemoproteus iwa*, described from *F. minor* from Tern and Laysan Islands, Hawaii (Work and Rameyer, 1996) and *Fregata magnificens* from Pacific Mexico (Madsen et al., 2007). The original description of *H. iwa* was based only on macrogametocytes from suboptimally preserved specimens with no molecular analyses, therefore necessitating a species redescription (Valkiūnas, 2005). This redescription was recently conducted during the assessment by Levin et al. (2011); further comparisons between the parasites are reported herein, and a redescription of *H. iwa* has been included. *Haemoproteus* spp. parasites are assigned to 2 different subgenera, *Haemoproteus* and *Parahaemoproteus*, which may differ in the type of host they infect (Valkiūnas, 2005). It has been proposed that parasites within the *Haemoproteus* subgenus are restricted to columbiform birds and use hippoboscids flies as vectors (Martinsen et al., 2008). *Haemoproteus iwa* has been assigned to the subgenus *Parahaemoproteus*, but based on a potential origin of the infection from columbiforms from Hawaii, from which the original description of the parasite species comes, it has been hypothesized that *H. iwa* may belong to the subgenus *Haemoproteus* (see comments in the species description in Valkiūnas, 2005). Indeed, Levin et al. (2011) assigned *H. iwa* to the subgenus *Haemoproteus*.

Here, we analyze *Haemoproteus* sp. from *F. andrewsi* and *F. minor* (Christmas Island) at the molecular and morphological level, as well as *F. magnificens* (Mexico) and 1 sample from *Fregata aquila* (Ascension Island). We also describe a new species of *Haemoproteus* from Christmas Island frigatebirds (*Fregata andrewsi*).

MATERIALS AND METHODS

Sample collection

Thirty-four *F. andrewsi* individuals were sampled on Christmas Island, Indian Ocean (10°25'S, 105°40'E), from July to September 2007 (5 samples), May 2008 (15 samples), and 2009 (14 samples). Additionally, we collected 29 samples from great frigatebirds (*F. minor*) breeding on the same island in 2009. Christmas Island frigatebirds were captured on their nests by hand, and great frigatebirds were captured at a feeding station. A drop of blood was obtained from the brachial vein and preserved in 100% ethanol for DNA analyses, while another drop of blood was immediately smeared, air-dried, fixed with 100% methanol, and later stained with Giemsa (1/10 v/v) for 30 min. Eleven samples from *F. magnificens* were collected on Isla Isabel situated in the Pacific Ocean (21°52'N, 105°54'W), about 70 km off the coast of the state of Nayarit, México, in 2001, and samples were stored in 1 ml of lysis buffer (EDTA 10 mM, Tris-Cl 10 mM, SDS 2%, pH = 8.0), but no smears were made. In addition, 1 blood sample from *F. aquila* was obtained from an individual captured while resting on a beach at Ascension Island situated in the Atlantic Ocean (7°56'S, 14°22'W). This sample was stored on an FTA card. Finally, 7

blood smears from *F. minor* positive for *Haemoproteus* sp., collected in Hawaii in 1994, were also analyzed by molecular methods.

DNA analyses

Blood samples preserved in ethanol were centrifuged at 13,000 g for 15 min. Afterward, the supernatants were discarded, and the pellets were resuspended in distilled water, frozen, and lyophilized to remove ethanol, and cored samples from the unique sample stored in an FTA card (Millipore) were transferred to collection vials. Genomic DNA from these samples was extracted using the following protocol: Dried samples were resuspended in 250 µl of SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH = 8), and SDS 20% (7 µl) and proteinase K (50 mg) were immediately added to the vials, maintaining the mix in an incubating shaker at 55 C overnight. The following day, ammonium acetate 4 M (250 µl) was added to the vials at room temperature for 30 min. Subsequently, vials were centrifuged at 13,000 g for 10 min. After removing the pellet, DNA was precipitated with ethanol and resuspended in sterile water (see Martínez et al. 2009; Quillfeldt et al. 2010). In the case of the samples preserved in the lysis buffer, proteinase K was added to 250 µl of each sample, the mix was incubated at 55 C overnight, and the DNA extraction was performed using the ammonium acetate precipitation as described previously. Cells from smears from Hawaiian frigatebirds were scraped using a sterile scalpel blade into Eppendorf tubes containing 70% ethanol. The vials were centrifuged at 13,000 g for 10 min, the supernatants discarded, and the pellets processed as described for the other samples.

Partial amplification of a 390-base-pair (bp) fragment of the *cytochrome b* (*cyt b*) gene of the parasites was accomplished by PCR using the non-specific primers PALU-F (5'-GGGTCAAATGAGTTTCTGG-3') and PALU-R (5'-DGGAAACAATATGTARAGGAGT-3') as described in Martínez et al. (2009). This set of primers is unable to distinguish between *Plasmodium* and *Haemoproteus* genera, so to confirm mixed infections formed by both *Haemoproteus* subgenera, the following forward primer PALU-F1 (5'-TAGTTAGCGACCCAACAC-3') was designed and used with the reverse primer PALU-R, to amplify specifically a DNA fragment of 301 bp from the *Haemoproteus* subgenera. The PCR conditions were similar to those selected for the first set of primers (Martínez et al., 2009); however, the annealing temperature had to be raised from 54 C to 60 C to avoid the amplification of *Parahaemoproteus* sp. DNA. Two procedures were performed to check the specificity of the new PCR: (1) Previous positive *Parahaemoproteus* sp. samples using primers PALU-F/PALU-R were checked to make sure they could not be amplified using the new primers, and (2) positive samples were digested with the endonuclease XbaI, as only the *Haemoproteus* subgenus possesses this restriction site. All amplicons digested with the endonuclease yielded 2 fragments of 170 and 131 bp (incubation for 5 hr at 37 C with 15 units of XbaI).

When the amplicons could not be sequenced due to scarcity of DNA, the samples were assigned to each subgenera according to the results achieved by PCR using the 2 set of primers (PALU-F/PALU-R and PALU-F1/PALU-R). The samples were assigned to: (1) *Parahaemoproteus* subgenus when the results were positive for the first set of primers and negative for the second set, (2) *Haemoproteus* subgenus when the results were the opposite (negative and positive, respectively), and (3) unknown when both sets of primers yielded a positive result.

To obtain a more informative phylogenetic analysis, we attempted to achieve longer amplicons (533 pb) using the primers 3760F (5'-GAG TGG ATG GTG TTT TAG AT-3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGT -3') as previously described (Beadell et al., 2004).

The DNA sequences corresponding to *Haemoproteus* sp. obtained from frigatebirds were aligned with those previously described by Santiago-Alarcón et al. (2010). In addition, other *Haemoproteus* lineages isolated from Columbiformes were included in the phylogenetic analyses in order to explore the relationship with these parasites supposedly belonging to the *Haemoproteus* subgenus. The alignment was performed using the CLUSTALW algorithm implemented in BIOEDIT program (Hall, 1999). Thereafter, the Gblocks program (Castresana, 2000; Talavera and Castresana, 2007) was used to establish informative 3' and 5' tails. The final alignment contained 95 taxa and 771 bp, including 2 lineages of *Leucocytozoon* sp. as the outgroup. The alignments were analyzed using Bayesian inference implemented in the MrBayes v3.2 program (Ronquist and Huelsenbeck, 2003), setting the substitution model to GTR. The model was previously selected using corrected AIC implemented in

TABLE I. Distribution of the 4 parasitic haplotypes on their hosts (*F.* = *Fregata*).

	<i>F.</i> <i>andrewsi</i>	<i>F.</i> <i>minor</i>	<i>F.</i> <i>aquila</i>	<i>F.</i> <i>magnificens</i>
<i>Parahaemoproteus</i> (FregPHae1)	5	2	0	2
<i>Parahaemoproteus</i> (FregPHae2)	2*	0	0	0
<i>Haemoproteus</i> (FregHae1)	3	10	1	3
<i>Haemoproteus</i> (FregHae2)	0	1	0	0

* One of them showed double peaks on the chromatogram indicating a mixed infection.

jModelTest 0.0.1 (Posada, 2008). The parameter gamma shape and the proportion of invariable sites were estimated by the program ("rates" was set as "invgamma"). Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses were run for 20 million generations and sampled every 1,000 generations. The rest of the parameters were selected according to their default options. At the end of the analysis, we set the burn-in period to 50%, where the chains reached stationary phase. The convergence of the parameters and topology were tested using the TRACER (Rambaut and Drummond, 2007) and AWTY (Wilgenbusch et al., 2004; Nylander et al., 2008) applications, respectively. In both cases, the statistical data and graphs did not show a lack of convergence. The consensus tree was estimated using 50% of the trees with a higher likelihood value. The sequence divergence between the different lineages was calculated with the Jukes-Cantor model implemented by the MEGA4 program (Tamura et al., 2007).

Morphometric measurements

Blood smears were scanned at $\times 100$ magnification using oil immersion (Merino et al., 1997). Intensity of infection was quantified per 2,000 or 10,000 erythrocytes (Godfrey et al., 1987). Parasite morphometric measurements were made with the aid of image analyzer software (Scion Image, Frederick, Maryland) from photos taken using $\times 100$ oil immersion and an Olympus BX41 microscope equipped with Olympus C5050 digital camera. Length and width of the parasite and parasite nucleus, along with the nuclear displacement ratio (NDR) and length and width of infected and uninfected erythrocytes and their nuclei, were measured for each parasite (Bennett and Campbell, 1972). Following Valkiūnas (2005), no measurements of areas were taken as they can be calculated from measured distances. Morphologic terminology followed Bennett and Peirce (1988) and Valkiūnas (2005). Comparison with previously published measurements from *H. iwa* was based on those of Levin et al. (2011).

Forty-four macrogametocytes and 37 microgametocytes in blood smears from 4 *F. andrewsi* individuals were measured (Table I). Average prevalence was 1.9 parasites per 2,000 erythrocytes (range 0.6–4). Differences between morphometric measurements of parasites were tested by a 2-tailed *t*-test for independent samples on the difference test module of Statistica 6.0 (Statsoft, Tulsa, Oklahoma).

RESULTS

By PCR, we detected parasites identified as *Haemoproteus* spp. in *F. andrewsi* (12/34), *F. magnificens* (6/11), *F. minor* (20/29), and *F. aquila* (1/1) (Table II). Two *Haemoproteus* haplotypes (FregHae1 and FregHae2) and 2 *Parahaemoproteus* haplotypes (FregPHae1 and FregPHae2) were detected (Fig. 1). Haplotypes FregHae1 and FregHae2 differed in only 1 nucleotide (0.3%). However, the genetic distance between the haplotypes FregPHae1 and FregPHae2 was 6.3% (see Table III). The distribution of the 4 haplotypes among frigatebird species is shown in Table I.

To explore morphological differences between parasite haplotypes and the original description of *H. iwa*, we selected blood smears from samples clearly infected by only 1 parasite lineage as

determined by the use of both sets of primers (Table IV). Four blood smears with a single *Parahaemoproteus* haplotype (FregPHae1) infecting *F. andrewsi* and sufficient macrogametocytes to compare with morphometrics of *H. iwa* were used to describe the new species.

Patent infections in blood smears corresponding to a single haplotype of the subgenus *Haemoproteus* (FregHae1) were found in *F. andrewsi* and *F. minor* from Christmas Island, and *F. aquila* (Table IV). Only the sample from *F. andrewsi* had sufficient mature gametocytes ($n = 20$) to allow an appropriate comparison with *H. iwa* or *H. valkiūnasi*. Only 1 microgametocyte was found in these infections, thus resembling *H. iwa*, which lacked microgametocytes in the original description (only 1 microgametocyte of 9 parasites in *F. minor*, 0 of 20 parasites in *F. andrewsi*, and 0 of 2 in *F. aquila*). Only 1 of the 2 blood smears corresponding to samples where the haplotype FregPHae2 (*Parahaemoproteus* subgenus) was genetically detected had enough gametocytes but, unfortunately, it had a mixed infection and was discarded for taxonomic purposes. We could not detect parasites in the other smear.

DESCRIPTION

Haemoproteus (Parahaemoproteus) valkiūnasi n. sp.

(Figs. 2–19, Table V)

Immature gametocytes (Figs. 2–4): Early stages most frequently lateral to erythrocyte nucleus. Parasite grows longitudinally to, but does not contact, host cell nucleus or membrane (Figs. 2–3). Gametocytes finally adhere to erythrocyte membrane (Fig. 4). Pigment granules, when observed, appear grouped near pole and are of small ($< 0.5 \mu\text{m}$) to medium size ($< 1.0 \mu\text{m}$) (Fig. 4). Margins uneven to slightly amoeboid.

Macrogametocytes (Figs. 5–10): Develop in mature erythrocytes. Cytoplasm stains blue, with granulated appearance; small vacuoles present and lacking valutin granules; staining clearly different from microgametocytes. Form halteridial, not circum-nuclear. Outline smooth varying to slightly sinuous. Parasite extends to poles of host cell (but see Fig. 10). Fully grown parasites appear closely appressed to host cell membrane. Parasite nucleus round in median or submedian location, not close to host cell nucleus. Pigment granules abundant, round, and of medium size (0.5 to 1.0 μm). Abundant (range 17–42) small and rod-shaped granules, evenly distributed across parasite cytoplasm, but can form clumps at extremes of parasites. Host cell nucleus displaced and may even contact host cell membrane (Fig. 8). Host cell nucleus can appear skewed (Fig. 9). Length of infected host cells significantly larger than uninfected erythrocytes (Table V; $P < 0.001$); erythrocyte nucleus of uninfected cells longer than those from cells infected by macrogametocyte (Table I; $P < 0.05$). Macrogametocytes longer than microgametocytes (Table V; $P < 0.003$), with shorter and wider nucleus (Table V; $P < 0.0001$ in both cases). Nuclear displacement ratio smaller (Table V; $P < 0.04$) and pigment granules more abundant (Table V; $P < 0.0001$) in macrogametocytes as compared to microgametocytes.

Microgametocytes (Figs. 11–18): Develop in mature erythrocytes. Cytoplasm slightly stained almost white with smooth outline, abundant (15–30), round, medium-size (0.5 to 1.0 μm) pigment granules with occasional smaller granules evenly distributed across parasite cytoplasm with occasional clumping at poles, small vacuoles, and lacking valutin granules. Halteridial in form, but not circum-nuclear (Figs. 13, 16). Fully grown parasites appear closely appressed to both host cell membrane or nucleus. Parasite nucleus elongated, slightly pink and indistinct in some cases with median to submedian location (97% of cases). Not close to host cell nucleus; host cell nucleus clearly displaced, but not in contact with host cell membrane. Host cell nucleus can appear tilted (Fig. 18). Lengths of host cells infected with microgametocytes significantly larger than those of uninfected cells (Table I; $P < 0.0001$). It is also possible to find double infections within the same erythrocyte (Fig. 19).

TABLE II. Summary of blood parasites found in frigatebirds in this and previous studies.

Species	Breeding colony site	N (ad)	N (chicks)	Parasites found	Method	Reference
Christmas Island frigatebird <i>Fregata andrewsi</i>	Christmas Island Christmas Island	9 34*		<i>Haemoproteus</i> (56%) <i>Parahaemoproteus</i> n. sp. <i>Haemoproteus</i> (<i>iva</i>) Total: 12 infected birds (35%)	PCR PCR	Quillfeldt et al. (2010) This study
Magnificent frigatebird <i>Fregata magnificens</i>	Offshore islands of northern Mexico Isla Isabel, Mexico Isla Isabel, Mexico	15 251 11		<i>Hepatozoon</i> sp. (7%) <i>Haemoproteus iva</i> (16%) <i>Parahaemoproteus</i> n. sp. 6 infected birds (55%) <i>Haemoproteus</i> (15%)	Blood smears Blood smears PCR	Clark and Swinehart (1969) Madsen et al. (2007) This study
Great/lesser frigatebird <i>Fregata minor</i> / <i>Fregata ariel</i>	Aldabra Atoll, Indian Ocean	–	62		Blood smears	Lowery (1971)
Great frigatebird <i>Fregata minor</i>	Genovesa, Galápagos Tern Island, French Frigate Shoals Laysan Island, Hawaii Christmas Island	24 20 40 29	10 20	<i>Haemoproteus</i> (29%) <i>Haemoproteus iva</i> (36%) <i>Haemoproteus iva</i> (35%) <i>Parahaemoproteus</i> n. sp. <i>Haemoproteus</i> (<i>iva</i>) Total: 20 infected birds (69%)	Blood smears Blood smears Blood smears PCR	Padilla et al. (2006) Work and Rameyer (1996) Work and Rameyer (1996) This study
Ascension frigatebird <i>Fregata aquila</i>	Ascension Island	1		<i>Haemoproteus</i> (<i>iva</i>)	PCR	This study

* Includes samples from Quillfeldt et al. (2010).

TABLE III. Sequence divergence between the different *Haemoproteus* (*H.*) haplotypes and other known *Haemoproteus* species. Analysis was conducted using the Jukes-Cantor model implemented in the program MEGA5. All ambiguous positions were removed for each sequence pair.

	<i>H. magnus</i>	<i>H. belopoloskyi</i>	<i>H. passeris</i>	<i>H. coatneyi</i>	<i>H. turtur</i>	<i>H. syrni</i>	<i>H. picae</i>	<i>H. sanguinis</i>	LIN13	FregPHae1	FregHae1	FregHae2
<i>H. belopoloskyi</i>	0.069											
<i>H. passeris</i>	0.062	0.069										
<i>H. coatneyi</i>	0.071	0.065	0.025									
<i>H. turtur</i>	0.084	0.080	0.064	0.069								
<i>H. syrni</i>	0.084	0.089	0.071	0.071	0.030							
<i>H. picae</i>	0.080	0.080	0.064	0.073	0.029	0.032						
<i>H. sanguinis</i>	0.071	0.076	0.046	0.053	0.062	0.064	0.062					
FregPHae1*	0.095	0.083	0.062	0.068	0.033	0.025	0.028	0.056		0.015		
LIN13†	0.101	0.092	0.071	0.080	0.039	0.036	0.033	0.056				
FregHae1	0.145	0.145	0.138	0.138	0.125	0.125	0.111	0.118	0.132	0.136		
FregHae2	0.157	0.157	0.150	0.150	0.136	0.136	0.122	0.122	0.138	0.142	0.003	
FregPHae2	0.089	0.069	0.031	0.031	0.050	0.037	0.050	0.031	0.066	0.063	0.107	0.111

* FregPHae1 haplotype was considered a new species (*Haemoproteus valkinnasi*).

† LIN13 corresponds to the *Haemoproteus* haplotype detected in *Larus crassirostris*.

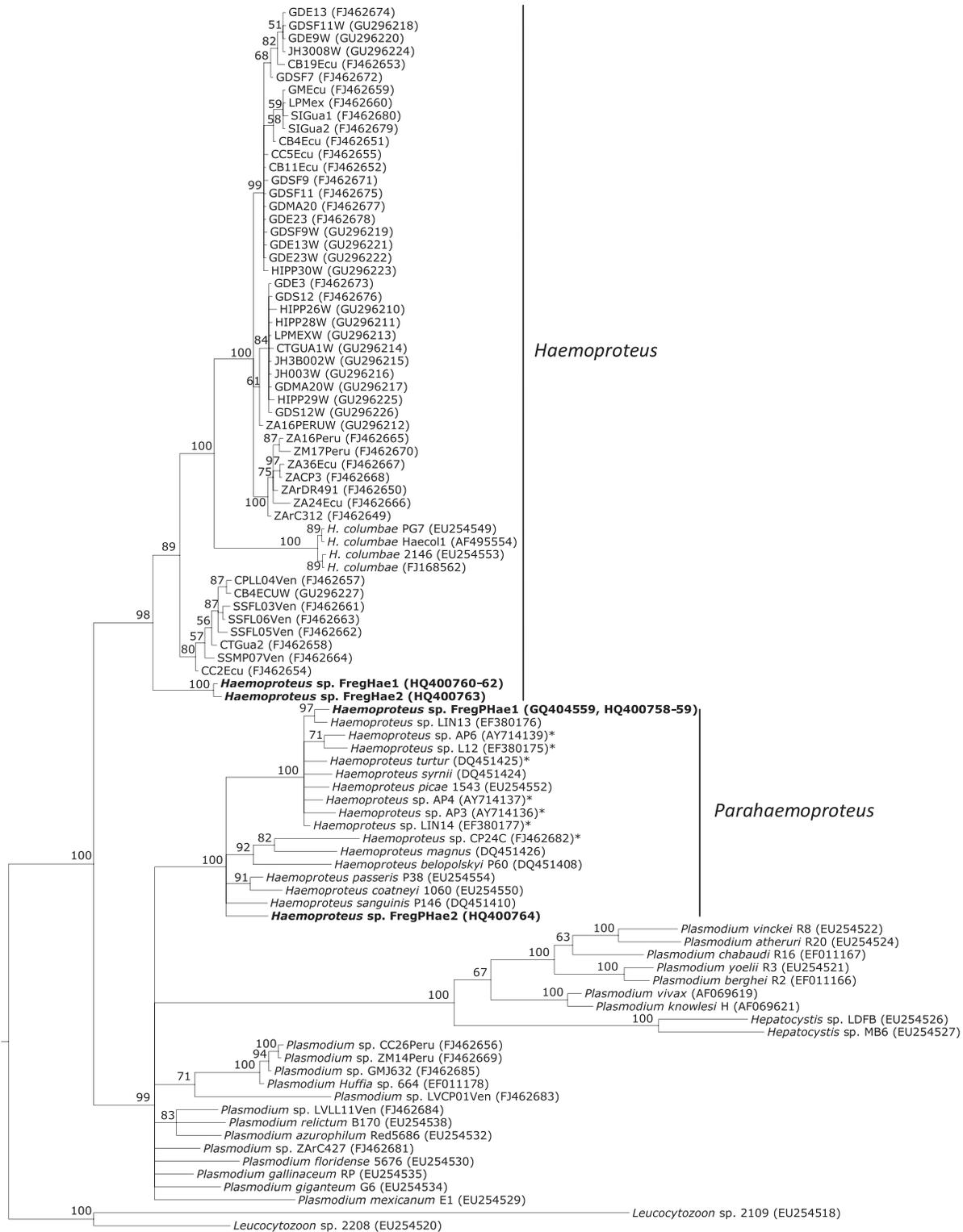


FIGURE 1. Phylogenetic inference of the *Haemoproteus* haplotypes found in frigatebirds (marked in bold). Phylogenetic tree was obtained with the program MrBayes v3.2 using the substitution model GTR, which was previously selected by means of jModeltest (see Methods). *Parahaemoproteus* lineages found by other authors in Columbiformes are marked with an asterisk.

TABLE IV. Results obtained by PCR using the 2 sets of primers (PALU-F/PALU-R and PALU-F1/PALU-R) for *Fregata* sp. The data for the *F. aquila* sample are not included in the Table because only PaluF/R primers were used.

	PCR (+/-)			
	PCR (+/+) unknown	<i>Parahaemo- proteus</i>	PCR (-/+) <i>Haemoproteus</i>	PCR (-/-) negative
<i>F. andrewsi</i>	3	8	1	22
<i>F. minor</i>	15	4	1	9
<i>F. magnificens</i>	3	3	0	5

Taxonomic summary

Type host: *Fregata andrewsi* L. (Pelecaniformes, Fregatiidae).

DNA sequences: Mitochondrial *cyt b* haplotype FregPHae1 (533 bp, GenBank accession no. GQ404559).

Type locality: Christmas Island, Indian Ocean, Australia; “Golf Course” breeding colony (10°25’S, 105°40’E, ~30 m above sea level).

Site of infection: Mature erythrocytes; no other data.

Prevalence: Overall prevalence in the Christmas Island frigatebirds was 5 of 9 (55.6%).

Distribution: Molecular evidence suggests that this parasite has a cosmopolitan distribution in frigatebirds.

Type specimens: Hapantotype (accession number ZMH PROTOZOA 313, intensity of parasitemia is 4 parasites/2,000 erythrocytes, *Fregata andrewsi*, Christmas Island, collected by Janos Hennicke in 2008) is deposited at the Zoologisches Museum, Universität Hamburg, Germany. Parahapantotypes (accession numbers: ZMH PROTOZOA 314, ZMH PROTOZOA 315, intensity of parasitemia is 1 parasites/2,000 erythrocytes and 2 parasites/2,000 erythrocytes, respectively, *Fregata andrewsi*, Christmas Island, collected by Janos Hennicke in 2008) are deposited at the Zoologisches Museum, Universität Hamburg, Germany. A voucher specimen was deposited at the International Reference Centre for Avian Haematozoa (IRCAH) in Queensland Museum, Australia, with reference number G465492.

Etymology: The species name is dedicated to Professor Gediminas Valkiūnas in recognition of his extensive and important work on avian blood parasite taxonomy.

Remarks

Haemoproteus valkiūnasi n. sp. individuals infecting *F. andrewsi* from Christmas Island had fewer pigment granules than *H. iwa* individuals infecting *F. minor* from Galapagos Islands (see Table V). In addition, in *H. valkiūnasi* individuals, there was separation of the parasite from the host cell, a shorter size, and a shorter NDR in microgametocytes. Similar to *H. iwa* from Hawaii, *H. valkiūnasi* individuals from Christmas Island seemed slightly amoeboid. The original descriptions of *H. iwa* lacked microgametocytes (Work and Rameyer, 1996), and we found that they were scant in samples with single infections by the FregHae1 haplotype from the *Haemoproteus* subgenus (1 microgametocyte and 8 macrogametocytes in 1 of the samples and no microgametocytes in the other 2). This haplotype is genetically identical to that detected in the samples used to redescribe *H. iwa* (Levin et al., 2011). Macrogametocytes corresponding to this haplotype (FregHae1) in *F. andrewsi* differ significantly from all the characteristics measured from *H. valkiūnasi* as expected, but, importantly, they also differ from *H. iwa* in *F. minor* (Levin et al., 2011) except for the width of host cell (data not shown). In addition, in parasites corresponding to FregHae1 in *F. andrewsi*, there is also separation of the parasite from host cell as occurs in *H. valkiūnasi*. These differences could be attributed to variation between infections in different host species, although the potential existence of undetected mixed infections in material used for redescription of *H. iwa* could also influence these results.

However, we also found significant differences in morphometrics between the infection by the haplotype FregPHae1 in *F. andrewsi* (corresponding to the description of *H. valkiūnasi*) and in *F. minor*, i.e., the macrogametocytes are longer (average \pm SD = $15.14 \pm 1.03 \mu\text{m}$, $n = 18$, $P < 0.0001$) and wider ($3.44 \pm 0.53 \mu\text{m}$, $n = 18$, $P = 0.001$), and the nuclear displacement ratio is smaller (0.75 ± 0.15 , $n = 18$, $P < 0.0001$)

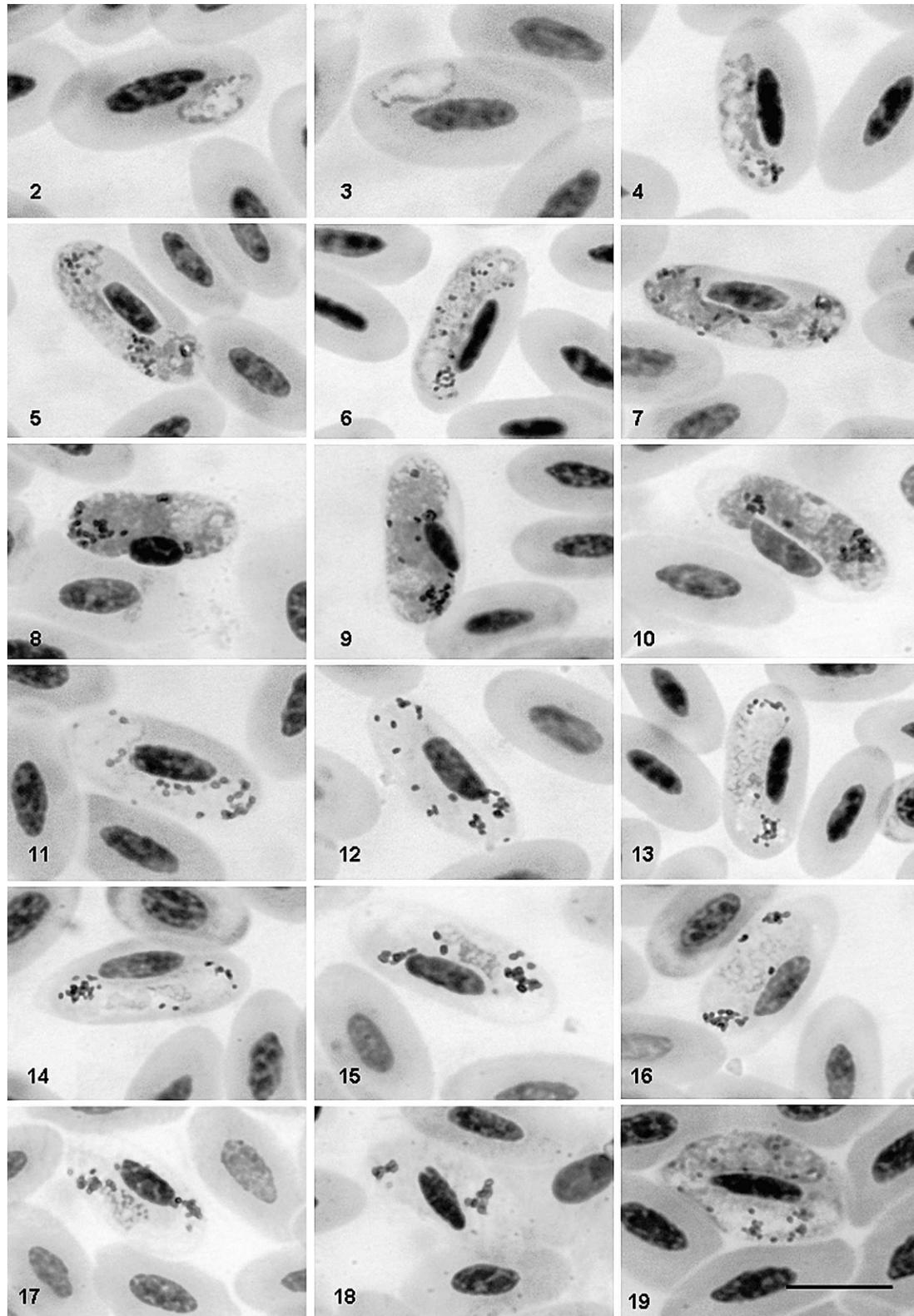
in parasites from *F. andrewsi*. Moreover, the microgametocytes are shorter (average \pm SD = $14.57 \pm 0.88 \mu\text{m}$, $n = 19$, $P = 0.005$) and have a shorter ($4.84 \pm 1.14 \mu\text{m}$, $n = 6$, $P = 0.01$) but wider ($2.89 \pm 0.88 \mu\text{m}$, $n = 6$, $P = 0.03$) nucleus in *F. minor*. Infections by this species also show an average longer nuclear displacement ratio ($0.66 \pm 0.12 \mu\text{m}$, $n = 19$, $P = 0.0001$) and have more pigment granules (28.8 ± 5.5 , $n = 19$, $P = 0.0001$). There are also some differences in the length of host cells between *F. minor* and *F. andrewsi*, which can partly explain these differences. The macrogametocytes of FregPHae1 from *F. minor* are still significantly different from those of *H. iwa*, except in measurements of the parasite nuclei. Microgametocytes only differ from *H. iwa* in being shorter and with fewer number of granules (data not shown).

The presence of identical sequences found in isolates from different frigatebird species across the world suggests the presence of parasite species with a broad geographic distribution. Unfortunately, we failed to amplify parasite DNA from blood smears collected in Hawaii where *H. iwa* was originally described (Work and Rameyer, 1996). Genetic divergence between FregPHae1 (*H. valkiūnasi*) and the closer *Haemoproteus* sequence in our phylogenetic analysis (LIN13 infecting *Larus crassirostris* from South Korea; see Fig. 1) was 1.5% (see Table III); however, the latter parasite has not been described morphologically. Although vectors of FregPHae1 have not been identified, it is logical to group it within the subgenus *Parahaemoproteus*, since phylogenetic relationships place it with other parasites in this subgenus and far from those in the *Haemoproteus* subgenus such as *H. columbae* (see Martinsen et al., 2008; Fig. 1, this work).

DISCUSSION

Haemoproteus species are not reported from Pelecaniformes, with the exception of frigatebirds. *Haemoproteus* sp. infections had been previously detected in *F. minor* from Hawaii (Work and Rameyer, 1996) and *F. magnificens* from Mexico (Madsen et al., 2007), both of which were infected by *H. iwa*. Also, *F. minor* and *F. ariel* from Aldabra Atoll, Indian Ocean (Lowery, 1971), and *F. minor* from Genovesa, Galapagos Islands (Padilla et al., 2006), were found to be infected with an unknown *Haemoproteus* species. More recently, Levin et al. (2011) redescribed *H. iwa* in *F. minor* from the Galapagos Islands. Following discovery of *Haemoproteus* sp. infections in Christmas Island frigatebirds (*F. andrewsi*; see Quillfeldt et al., 2010), we conducted a genetic study on infection by different haplotypes of *Haemoproteus* in 4 frigatebird species, 1 of them (*F. aquila*) never sampled before (Table II). Our results indicate that blood parasites within *Haemoproteus* commonly infect frigatebirds throughout their range (Table II).

The haplotypes FregHae1 and FregHae2 are clearly classed with the *Haemoproteus* subgenus (see Fig. 1) and probably belong to the same species due to the low genetic distance between them (0.3%). One of these sequences (FregHae1) corresponds to those used in redescription of *H. iwa* by Levin et al. (2011). The lack, or very low number, of microgametocytes present in FregHae1 infections also suggests that it corresponds to *H. iwa*, because this species also lacks microgametocytes in its original description (Work and Rameyer, 1996). Although we found significant differences in morphometric characters between *H. iwa* and infections by FregHae1 in *F. andrewsi*, these can be attributed to both the fact that the original description comes from infections in a different host species (*F. minor*), and the possibility that the redescription of *H. iwa* includes mixed infections with other undetected *Haemoproteus* lineages because authors did not check for the presence of these infections. In any case, it is clear that the distribution range of *H. iwa* (FregHae1) is cosmopolitan, parasitizing 4 frigatebird species from Indonesia, the eastern Pacific, and the Atlantic Ocean.



FIGURES 2–19. *Haemoproteus (Parahaemoproteus) valkūnasi* from the blood of the Christmas Island frigatebird *Fregata andrewsi*. (2–4) Young gametocytes. (5–10) Macrogametocytes. (11–18) Microgametocytes. (19) Double infection by gametocytes of both sexes. Giemsa-stained thin blood films. Bar = 10 μ m.

TABLE V. Morphometric characters of gametocytes and host cells of *Haemoproteus valkii*. All sizes are given in micrometers (SD = standard deviation). Data for *Haemoproteus iwa* come from Levin et al. (2011). Values in bold indicate significant differences with values from *H. iwa* at $P < 0.05$ calculated by means of 2-tailed t -test for independent samples.

Feature	<i>H. iwa</i>				<i>H. valkii</i>			
	n	Range	Mean	SD	n	Range	Mean	SD
Uninfected erythrocyte	21				32			
Length		14.3–16.6	15.2	0.5		13.4–17.4	15.5	0.9
Width		6.6–8.4	7.6	0.5		6.7–8.9	7.8	0.5
Length of nucleus		6.1–8.2	7.0	0.5		5.8–7.7	6.7	0.5
Width of nucleus		2.1–3.7	2.6	0.4		1.9–3.3	2.6	0.3
Erythrocyte parasitized by macrogametocyte	21				44			
Length		13.2–17.7	16.2	1.2		14.3–19.3	17.3	1.2
Width		6.9–10.2	8.3	0.9		5.4–9.6	7.8	0.8
Length of nucleus		5.7–7.3	6.8	0.4		4.6–7.9	6.4	0.7
Width of nucleus		2.1–2.7	2.3	0.1		1.7–3.3	2.5	0.3
Erythrocyte parasitized by microgametocyte	21				37			
Length		13.0–18.0	15.3	1.5		15.3–19.4	17.3	1.1
Width		7.1–11.0	8.5	0.9		6.4–9.5	8.1	0.7
Length of nucleus		6.1–8.2	7.2	0.6		5.3–7.8	6.6	0.6
Width of nucleus		1.9–2.9	2.3	0.3		1.8–3.3	2.5	0.3
Macrogametocyte	21				44			
Length		15.5–19.6	17.9	1.1		13.2–19.0	16.7	1.3
Width		3.3–5.7	4.3	0.6		2.7–5.4	4.0	0.6
Length of nucleus		2.6–4.4	3.5	0.5		2.5–7.4	3.8	1.1
Width of nucleus		1.8–3.4	2.4	0.4		1.5–4.1	2.8	0.6
NDR		0.2–0.5	0.4	0.1		0–0.8	0.4	0.2
No. of pigment granules		49–67	57.4	5.1		17–42	29.4	5.1
Microgametocyte	21				37			
Length		14.6–20.9	17.3	1.6		12.7–19.0	15.7	1.5
Width		3.0–4.2	3.5	0.3		2.5–5.7	3.9	0.6
Length of nucleus*		—	—	—		3.8–10.3	6.8	1.8
Width of nucleus*		—	—	—		0.8–3.8	2.1	0.8
NDR		0.5–0.9	0.7	0.1		0.2–0.8	0.5	0.2
No. of pigment granules		25–40	31.7	3.4		15–30	23.1	4.3

* n = 34.

It was initially proposed that *H. iwa* could be related to haemoproteids infecting columbiform birds because this was the only other bird in Hawaii infected by *Haemoproteus* (Valkiūnas, 2005). Although haemoproteids infecting columbiforms were recently assigned to the subgenus *Haemoproteus* (Beadell et al., 2004; Ishtiaq et al., 2007; Martinsen et al., 2008; Santiago-Alarcón et al., 2010), this clearly does not apply to all Columbiformes (see phylogenies presented in Martinsen et al., 2008; Santiago-Alarcón et al., 2010; and this study, Fig. 1). In addition, the FregHae1 and FregHae2 haplotypes form a basal sister group within the *Haemoproteus* subgenus cluster, suggesting that parasites from frigatebirds and Columbiformes within this subgenus shared a common ancestor but followed independent evolutionary ways. In other words, the hypothesis of a jump from Columbiformes to frigatebirds in Hawaii Island proposed by Valkiūnas (2005) is not supported, although these parasite haplotypes are clearly within the subgenus *Haemoproteus*. It is also not supported by the biology, since columbiforms are mainly terrestrial species, whereas frigatebirds are pelagic, and, thus, opportunities for cross-species transmissions or contract would seem minimal.

The haplotype FregPHae1 was isolated from *F. andrewsi*, *F. minor*, and *F. magnificens*. Although it was not isolated from *F. aquila* (where only 1 sample was available), this haplotype can also be considered as cosmopolitan. Morphometrics of parasites bearing the FregPHae1 haplotype were sufficiently different than known species of *Haemoproteus* to justify their description as a new and distinct species, *H. valkiūnasi*. This species is clearly grouped along with *Haemoproteus* species within the subgenus *Parahaemoproteus* infecting non-passerine birds (Fig. 1), including some columbiforms. The fact that several *Haemoproteus* infections of columbiform birds have not been phylogenetically grouped within the subgenus *Haemoproteus* could be justified if host-parasite coevolution for these parasites follows the definitive hosts (the invertebrate) where sexual reproduction of the parasite takes place, as has been suggested for other apicomplexans (Šlapeta et al., 2003), and not the intermediate host (the bird). In that case, the *Haemoproteus* subgenus should be restricted to species transmitted by hippoboscids as definitive hosts (Valkiūnas, 2005) and not to columbiform birds, as previously suggested (Santiago-Alarcón et al., 2010). In this respect, identification of vectors and morphometric description of *Haemoproteus* lineages phylogenetically close to parasites in the subgenus *Parahaemoproteus* that infect columbiform birds (see Fig. 1) are clearly needed.

The phylogenetic analysis here (Fig. 1) shows that haplotype FregPHae1 (*H. valkiūnasi*) is strongly grouped with a *Haemoproteus* LIN13 haplotype infecting *L. crassirostris* from South Korea (Ishtiaq et al., 2007). This evolutionary relationship, added to the fact that gulls and frigatebirds are commonly infected by haemoproteids (Quillfeldt et al., 2010), suggests that *Parahaemoproteus* species infecting seabirds share a common ancestor. However, the haplotype FregPHae2 isolated from *F. andrewsi* was also included within the *Parahaemoproteus* cluster, although phylogenetically distant not only from *Haemoproteus* LIN13 and *Haemoproteus* FregPHae1 (*H. valkiūnasi*), but even from *Haemoproteus* species isolated from non-passerine birds (Fig. 1). The genetic distance between haplotype FregPHae2 and FregPHae1 (*H. valkiūnasi*) was 6.3% (Table III), and thus they probably belong to different species. The low prevalence of haplotype

FregPHae2, together with its phylogenetic relationship with haplotypes isolated from passerine birds, could be indicative of a host-switching event. Future phylogenetic analysis including more *Haemoproteus* haplotypes isolated from passerine birds on Christmas Island could help to test this hypothesis. In any case, it is clear that frigatebirds are infected by several parasite haplotypes of the genus *Haemoproteus*, with mixed infections being relatively frequent. The identification and description of all these potential species of parasites, as well as their evolutionary origins and relationships, merit further research.

ACKNOWLEDGMENTS

The samples of Christmas Island (CI) birds were collected within the framework of the Christmas Island Seabird Project (www.seabirdproject.cx), which was supported by the University of Hamburg Research Fund, Christmas Island Tourist Association, Island Explorer Holidays, CI Territory Week Committee, and CI Island Care. Fieldwork was carried out under permission of Parks Australia North Christmas Island and Darwin and was approved by the Animal Ethics Committee of the Charles Darwin University, Darwin, Australia. We thank Parks Australia North Christmas Island for their support in all aspects of the study, and N. Dehnhard, K. Flachsbath, L. Braun, J. Navarro, and M. van der Stap for their help in the field. During the preparation of this work, J.M. and S.M. were supported by project CGL2009-09439 from the Spanish Ministry of Science and Technology, and P.Q. was supported by Deutsche Forschungsgemeinschaft (DFG) (Qu 148/3). Samples from *F. magnificens* from the National Park Isla Isabel, Mexico, were collected with permission from SEMARNAT and with logistic support from the staff of the park and the Armada de México. A. J. Nieto and L. Lee greatly helped during fieldwork, and financial support granted to Jose Luis Osorno and R. Torres was provided by the Mexican Ministry of Science and Technology (CONACYT 34899-V). Thanks are due to Gustavo Tomás for his help and advice. We also thank the Ascension Island Government conservation team for their help: Olivia Renshaw, Natasha Williams, Nathan Fowler, and Dane Wade, and RSPB, U.K. Mention of products or trade names does not imply endorsement by the U.S. government.

LITERATURE CITED

- BEADELL, J. S., E. GERING, J. AUSTIN, J. P. DUMBACHER, M. A. PEIRCE, T. K. PRATT, C. A. ATKINSON, AND R. C. FLEISCHER. 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology* **13**: 3829–3844.
- BENNETT, G. F., AND G. CAMPBELL. 1972. Avian Haemoprotidae. I. Description of *Haemoproteus fallisi* n. sp. and a review of the haemoproteids of the family Turdidae. *Canadian Journal of Zoology* **50**: 1269–1275.
- , AND M. A. PEIRCE. 1988. Morphological form in the avian haemoprotidae and annotated checklist of the genus *Haemoproteus* Kruse, 1890. *Journal of Natural History* **22**: 1683–1696.
- BIRD LIFE INTERNATIONAL. 2011. IUCN Red List for birds. Available at: <http://www.birdlife.org>. Accessed 25 February 2011.
- CASTRESANA, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540–552.
- CLARK, G. W., AND B. SWINEHART. 1969. Avian haematozoa from the offshore islands of northern Mexico. *Bulletin of the Wildlife Disease Association* **5**: 111–112.
- GODFREY, R. D., A. M. FEDYNICH, AND D. B. PENCE. 1987. Quantification of hematozoa in blood smears. *Journal of Wildlife Diseases* **23**: 558–565.
- HALL, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.
- ISHTIAQ, F., E. GERING, J. H. RAPPOLE, A. R. RAHMANI, Y. V. JHALA, C. J. DOVE, C. MILENSKY, S. L. OLSON, M. A. PEIRCE, AND R. C. FLEISCHER. 2007. Prevalence and diversity of avian hematozoan parasites in Asia: A regional survey. *Journal of Wildlife Diseases* **43**: 382–398.
- IUCN. 2010. IUCN Red List of Threatened Species. Version 2010.3. Available at: www.iucnredlist.org. Accessed 04 October 2010.
- LEVIN, I. I., G. VALKIŪNAS, D. SANTIAGO-ALARCÓN, L. L. CRUZ, T. A. IEZHOVA, S. L. O'BRIEN, F. HAILER, D. DEARBORN, E. A. SCHREIBER,

- R. C. FLEISCHER, ET AL. 2011. Hippoboscid-transmitted *Haemoproteus* parasites (Haemosporida) infect Galapagos Pelecaniform birds: Evidence from molecular and morphological studies, with a description of *Haemoproteus iwa*. *International Journal for Parasitology* **41**: 1019–1027.
- LOWERY, R. S. 1971. Blood parasites of vertebrates on Aldabra. *Philosophical Transactions of the Royal Society of London, Series B* **260**: 577–580.
- MADSEN, V., G. VALKIŪNAS, T. A. IEZHOVA, C. MERCADE, M. SÁNCHEZ, AND J. L. OSORNO. 2007. Testosterone levels and gular pouch coloration in courting magnificent frigatebird (*Fregata magnificens*): Variation with age-class, visited status and blood parasite infection. *Hormones and Behavior* **51**: 156–163.
- MARTÍNEZ, J., J. MARTÍNEZ-DE-LA-PUENTE, J. HERRERO, S. DEL CERRO, E. LOBATO, J. RIVERO-DE-AGUILAR, R. A. VÁSQUEZ, AND S. MERINO. 2009. A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in birds: On the inefficiency of general primers for detection of mixed infections. *Parasitology* **136**: 713–722.
- MARTINSEN, E. S., S. L. PERKINS, AND J. J. SCHALL. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* **47**: 261–273.
- MERINO, S., J. POTTI, AND J. A. FARGALLO. 1997. Blood parasites of some passerine birds from central Spain. *Journal of Wildlife Diseases* **33**: 638–641.
- NYLANDER, J. A., J. C. WILGENBUSCH, D. L. WARREN, AND D. L. SWOFFORD. 2008. AWTY (are we there yet): A system for graphical exploration of MCMC convergence in Bayesian phylogenetics. *Bioinformatics* **24**: 581–583.
- ORTA, J. 1992. Family Fregatidae (Frigatebirds). *In Handbook of the birds of the world, Vol. 1*, J. del Hoyo, A. Elliot, and J. Sargatal (eds.). Lynx Edicions, Barcelona, Spain, p. 362–374.
- OSORNO, J. L., R. TORRES, AND C. MACÍAS-GARCÍA. 1992. Kleptoparasitic behavior of magnificent frigatebird: Sex bias and success. *Condor* **94**: 692–698.
- PADILLA, L. R., N. K. WHITEMAN, J. MERKEL, K. P. HUYVERT, AND P. G. PARKER. 2006. Health assessment of seabirds on Isla Genovesa, Galápagos. *Ornithological Monographs* **60**: 86–97.
- POSADA, D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.
- QUILLFELDT, P., J. MARTÍNEZ, J. HENNICKE, K. LUDYNIA, A. GLADBACH, J. F. MASELLO, S. RIOU, AND S. MERINO. 2010. Haemosporidian blood parasites in seabirds. A comparative genetic study of species from Antarctic to tropical habitats. *Naturwissenschaften* **97**: 809–817.
- RAMBAUT, A., AND A. J. DRUMMOND. 2007. Tracer v1.4. Available from: <http://beast.bio.ed.ac.uk/Tracer>. Accessed 24 January 2010.
- RONQUIST, F., AND J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
- SANTIAGO-ALARCÓN, D., D. C. OUTLAW, R. E. RICKLEFS, AND P. G. PARKER. 2010. Phylogenetic relationships of haemosporidian parasites in New World Columbiformes, with emphasis on the endemic Galapagos dove. *International Journal for Parasitology* **40**: 463–470.
- ŠLAPETA, J. R., D. MODRÝ, J. VOTÝPKA, M. JIRKŮ, J. LUKEŠ, AND B. KOUDELA. 2003. Evolutionary relationships among cyst-forming coccidian *Sarcocystis* spp. (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle. *Molecular Phylogenetics and Evolution* **27**: 464–475.
- TALAVERA, G., AND J. CASTRESANA. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**: 564–577.
- TAMURA, K., J. DUDLEY, M. NEI, AND S. KUMAR. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.
- VALKIŪNAS, G. 2005. Avian malaria parasites and other Haemosporidia. CRC Press, Boca Raton, Florida, 932 p.
- WILGENBUSCH, J. C., D. L. WARREN, AND D. L. SWOFFORD. 2004. AWTY: A system for graphical exploration of MCMC convergence in Bayesian phylogenetic inference. Available from: <http://ceb.csit.fsu.edu/awty>. Accessed 25 January 2010.
- WORK, T. M., AND R. A. RAMEYER. 1996. *Haemoproteus iwa* n. sp. in great frigatebirds (*Fregata minor* [Gmelin]) from Hawaii: Parasite morphology and prevalence. *Journal of Parasitology* **82**: 489–491.