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ORIGINAL PAPER

Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: Plasmodiidae), *Plasmodium* (*Novyella*) *homopolare* sp. nov.

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Abstract *Plasmodium* (*Novyella*) *homopolare*, a newly described *Plasmodium* species, was found in a wide range of Passeriformes species in California, USA, and Colombia. This parasite infected more than 20 % of the sampled bird community ($N = 399$) in California and was found in 3.6 % of birds sampled ($N = 493$) in Colombia. Thus far, it has been confirmed in North and South America where it is present in numerous species of migratory and resident birds from six families. Based on 100 % matches, or near-100 % matches (i.e., ≤2-nucleotide difference), to DNA sequences previously deposited in GenBank, this parasite is likely also distributed in the Eastern USA, Central America, and the Caribbean. Here, we describe the blood stages of *P. homopolare* and its mtDNA cytochrome *b* sequence. *P. homopolare* belongs to the subgenus *Novyella* and can be readily distinguished from the majority of other *Novyella* species, primarily, by the strictly polar or subpolar position of meronts and advanced trophozoites in infected erythrocytes. We explore possible reasons why this widespread parasite has not been described in earlier studies. Natural malarial parasitemias are usually light and co-infections predominate, making the parasites difficult to detect and identify to species when relying exclusively on microscopic examination of blood films. The combined application of sequence data and digital microscopy techniques, such as those used in this study, provides identifying markers that will facilitate the diagnosis of this parasite in natural avian populations. We also address the evolutionary relationship of this parasite to other species of *Plasmodium* using phylogenetic reconstruction.

Keywords *Plasmodium* · Avian malaria · Hematozoa · Morphospecies · California · Colombia

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Introduction

Plasmodium is a genus of apicomplexan (Apicomplexa, syn. Sporozoa) parasites within the order Haemosporida. *Plasmodium* species cause avian malaria (as well as mammalian and reptilian malarias) and affect a wide range of wild birds in most parts of the world (Valkiūnas 2005). Approximately 50 species of avian *Plasmodium* have been described to date, which vary widely in geographic distribution, pathogenicity, and host specificity (Atkinson 2008). *Plasmodium* parasites are closely related to haemosporidian parasites of the genera *Haemoproteus* and *Leucocytozoon*. While species of the three genera differ in morphology and certain developmental characteristics, they share a complex life cycle requiring a susceptible vertebrate host for asexual reproduction and a competent

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dipteran vector for sexual reproduction and sporogony (Garnham 1966; Valkiūnas 2005; Atkinson 2008).

The study and analysis of *Plasmodium* sp.-vector-host systems require proper species identification and a resolved taxonomy of *Plasmodium* species (Valkiūnas et al. 2008a; Ilgūnas et al. 2013; Mantilla et al. 2013a). While polymerase chain reaction (PCR) has greatly increased our understanding of the range and genetic diversity of these parasites, few morphospecies have been formally described, even among well-studied avian hosts, despite the increasing number of nucleotide sequences deposited in GenBank (Valkiūnas et al. 2008a). Identification of *Plasmodium* morphospecies requires detailed microscopy and a high degree of competence to distinguish these parasite species from one another in various life stages, which is particularly difficult in cases of light parasitemias, which predominate in wildlife. Consequently, comprehensive taxonomic identifications are seldom done, misidentifications are common, and much work remains to determine the geographic ranges, phylogenetic relationships, and host and vector distributions of avian *Plasmodium* species (Valkiūnas et al. 2008b). The combined application of PCR and microscopy has made some headway in facilitating the recognition of new haemosporidian parasite lineages, especially those that appear to be cryptic (Sehgal et al. 2006; Valkiūnas et al. 2010; Levin et al. 2012; Mantilla et al. 2013b). The development of the MalAvi database also aimed to help address this knowledge gap by providing a single database for avian blood parasites of the *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* genera that have been amplified using a specified portion of the cytochrome *b* (cyt*b*) sequence (Bensch et al. 2009). However, biological questions such as host and vector specificity and competency must be answered to provide a more complete picture of the diversity of parasite lineages and relationships.

Here, we utilize microscopy and genetic sequencing to describe and name a New World species of *Plasmodium*, *Plasmodium homopolare* sp. nov., which has been identified from hosts in Colombia and California, USA, and, based on DNA sequences, has likely distributions in the Eastern USA, Central America, and the Caribbean. We also address the evolutionary relationship of this parasite to other species of *Plasmodium* using phylogenetic reconstruction. Finally, we explore some possible reasons why this apparently widespread parasite has not been described in earlier studies.

Materials and methods

Ethical statement

The bird sampling methods in California were approved by the Institutional Animal Care and Use Committee (IACUC). Sample collection was performed under permits supplied by the US Geological Survey Bird Banding Laboratory and a Scientific Collecting Permit issued by the California Natural Resources Agency, Department of Fish and Game. The bird sampling methods in Colombia were approved by the Comité de Bioética of Departamento de Ciencias para la Salud Animal of Facultad de Medicina Veterinaria y de Zootecnia (act 005 of 2010). Sample collection was performed under permits supplied by the Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales de Colombia (UAESPNN) Subdirecciónténcica and Autoridad Nacional de Licencias Ambientales (ANLA).

Study sites

Sampling of the California birds took place over 38 trapping days between April 2011 and January 2013, during the months of April, May, June, October, and January. Trapping took place at China Creek County Park, a 120-ac riparian habitat in the southern Central Valley of California, approximately 20 mi east of the city of Fresno (36° 44′ N, 119° 29′ W, 120 m above sea level). The site is located in the King's River watershed of the Sierra Nevada mountain range and includes a small stream and two ponds, as well as areas of oak grassland.

In Colombia, birds were sampled over 58 trapping days in four periods: April 2011, between June and July 2011, between December 2011 and January 2012, and December 2012. Sampling took place at Los Nevados National Natural Park (04° 43′ N, 75° 27′ W, 3,300 m above sea level) and two localities inside Ucumari Natural Regional Park (04° 42′ N, 75° 32′ W; 04° 42′ N, 75° 29′ W, 2,400 m above sea level). These localities included Andean and high Andean forests as well as small areas of grasslands used for cattle raising, several small streams, and the Otun River crossing the study sites.

Samples and blood film examination

We obtained 25–50 μl of blood from each bird via brachial venipuncture and prepared two blood smears using a drop of blood on each slide. Slides were air-dried and fixed in absolute methanol for 1–5 min and stained with Giemsa as described by Valkiūnas (2005). Remaining blood from California samples was placed in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 2 % SDS) and stored at ambient temperature while in the field and then preserved at −80 °C upon returning to the laboratory. For the Colombian birds, whole blood was stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) and stored at ambient temperature while in the field and then preserved at −20 °C in the laboratory.

Morphometrics and the species description were based on blood smears and performed using an Olympus BX51 light microscope equipped with Olympus DP12 digital camera and imaging software. Olympus DP-SOFT was used to examine slides, to prepare illustrations, and to take measurements from

Californian samples. Blood smears from Colombian birds were examined using a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland), and digital images were prepared using the Leica EC3 digital camera and processed with LAS EZ software (Leica Microsystems Switzerland Limited 2012). Measurements were taken from digital images using ImageJ (Schneider et al. 2012). For both Californian and Columbian smears, approximately 100–150 fields were examined at low magnification (×400), and then, at least 100 fields were studied at high magnification (×1,000). Parasitemia was estimated based on the number of infected erythrocytes per 10,000 cells examined, by counting 100 fields at a magnification of ×1,000 in areas of the slide where the blood cells formed a monolayer. A single monolayer field contained approximately 100 cells under ×1,000 magnification (Muñoz et al. 1999). To determine the possible presence of co-infections with other haemosporidian parasites in the type material of new species, the entire blood films from hapantotype and parahapantotype series were examined microscopically at low magnification. The morphometric features studied were those defined by Valkiūnas (2005). Student's *t* test for independent samples was used to determine statistical significance between mean linear parameters. A *P* value of 0.05 or less was considered significant.

DNA extraction, PCR amplification, and sequencing

California samples

Parasite DNA was extracted from whole blood stored in lysis buffer following animal tissue protocols recommended for the Wizard SV Genomic DNA Purification kits (Promega Corporation, Madison, WI). Success of each DNA extraction was verified with primers that amplify the brain-derived neurotrophic factor (BDNF) (Sehgal and Lovette 2003). *Plasmodium* spp. were detected by nested PCRs that amplify sections of the mitochondrial cyt*b* gene. The primers used for the first and second reactions were HaemNF/HaemNR2 and HaemF/HaemR2, respectively and have been described previously (Waldenström et al. 2004).

All PCR reactions were carried out in 25-μl reactions and were accompanied by negative controls (ddH_2O) and positive controls (samples from infected birds previously confirmed by sequencing and microscopy) to control for any contamination and to confirm success of the PCR. The resulting PCR products were run out on a 1.8 % agarose gel using 1× TBE and visualized by ethidium bromide staining under ultraviolet light to check for positive infections.

PCR products from positive infections were purified using ExoSap following the manufacturer's instructions (United States Biochemical Corporation, Cleveland, OH). Bidirectional sequencing of the PCR fragments was performed using the BigDye® version 1.1 sequencing kit (Applied Biosystems, Inc., Foster City, California) and an ABI Prism 3100 automated sequencer (Applied Biosystems, Inc., Foster City, CA).

Colombian samples

DNA extraction was made only from infected blood samples diagnosed by microscopy. DNA was extracted by the phenol-chloroform method (Sambrook et al. 1989). A fragment of the cyt*b* gene was amplified using the nested PCR protocol described by Hellgren et al. (2004), modified as follows: first reaction with total volume of 12.5 μl included 50-ng DNA template, 0.6 mM of each primer (HaemNFI/HaemNR3), and 5.624 μl of GoTaq® Green Master Mix (Promega, USA). The thermal profile of the first reaction consisted of an initial step of 94 °C for 3 min; followed by 5 cycles with denaturation at 94 °C for 1 min, annealing at 45 °C for 1.5 min, and extension at 72 °C for 1.5 min; followed by 25 cycles with denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s; and followed by a final extension at 72 °C for 10 min. The second PCR reaction in 25-μl volumes included 2 μl of DNA template from the previous PCR, 0.6 mM of each primer (HAEMF/HAEMR2), and 11.249 μl of GoTaq® Green Master Mix (Promega, USA). The second thermal profile was conducted as described by Hellgren et al. (2004) except that an annealing temperature of 52 °C was used instead of 50 °C.

The amplification product was evaluated by running 2 μl of the final PCR product on a 2 % agarose gel. Every reaction was accompanied by negative controls (ddH_2O) and positive controls (samples from infected birds as confirmed by sequencing and microscopy); no false positives or negatives were recorded. Amplified products were precipitated with ammonium acetate and 95 % ethanol (Bensch et al. 2000) and sequenced in both directions in a 3730xl DNA analyzer (Applied Biosystems, Inc., Foster City, California) by Macrogen, Inc.

Phylogenetic analysis

Sequences were edited using Sequencher 4.8 (GeneCodes, Ann Arbor, MI) and aligned using SEAVIEW software (Galtier et al. 1996). In addition to *P. homopolare*, our analysis included reference sequences from GenBank that have well-established positive morphological identifications (Valkiūnas 2005; Bensch et al. 2009). *Leucocytozoon* sp. LSISKIN2 (accession no. AY393796) served as the out-group.

The appropriate model of sequence evolution was determined by the software MrModeltest (Nylander et al. 2004) to be GTR+Γ. Phylogenetic reconstruction was done using Bayesian Inference in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) using the appropriate models of sequence evolution described above. Two Markov Chain Monte Carlo (MCMC) simulations were run simultaneously for 10 million generations with sampling every 200 generations, generating 50,000 trees. The first 12,500 trees were discarded from the

sample as the "burn-in" period, accounting for 25 % of the trees. The remaining 37,500 trees were used to construct a majority rule consensus tree and to calculate the posterior probabilities of the individual clades.

The sequence divergence between the different lineages was calculated using the uncorrected *p* distance, implemented in the program PAUP (Swofford 2001).

Results

Of 399 birds sampled in our California study, 31 % harbored a *Plasmodium* infection. The majority, 68 %, of those infected with *Plasmodium* were infected with *P. homopolare*. This suggests that more than one in five birds in our study area were infected with this species. We found that *P. homopolare* was a host generalist, infecting nine different passerine species across five families at the California field site, but 80 % of these infected birds were from the Emberizidae family. The Spotted towhee (*Pipilo maculatus*) and Song sparrow (*Melospiza melodia*), both resident emberizids, had infection prevalences approaching 70 %.

P. homopolare primarily infected resident birds at the California study site. Four individuals aged as hatch year (HY) birds were infected, which indicates that the parasite is transmitted locally. In addition, six individuals representing three migratory species—Lincoln's sparrow (*Melospiza lincolnii*), Fox sparrow (*Passerella iliaca*), and Wilson's warbler (*Cardellina pusilla*)—were also infected (Table 1). All of these migrants were after-hatch year (AHY) birds.

Of 493 birds sampled in Colombia, 3.6 % were infected with *P. homopolare*. This parasite was found infecting birds of three families: 67 % of birds infected were from the Emberizidae, 22 % from the Parulidae, and 11 % from the Thraupidae (Table 1). *P. homopolare* was found in six passerine species, with prevalences between 3.8 and 25 %: White-naped brush finch (*Atlapetes albinucha*), Rufous-collared sparrow (*Zonotrichia capensis*), Russet-crowned warbler (*Myiothlypis coronatus*), Golden-fronted whitestart (*Myioborus ornatus*), Blue-winged mountain tanager (*Anisognathus somptuosus*), and Yellow-throated bush tanager (*Chlorospingus flavigularis*). All infected hosts were resident birds of Colombia. Although the boreal migrant species Blackburnian warbler (*Setophaga fusca*) was captured (data are not shown), none was infected with *P. homopolare*.

GenBank queries revealed that the California and Colombia sequences matched 100 % with two previously deposited sequences in GenBank: AF465555 and JN819334. These sequences were isolated from the Silver-throated tanager (*Tangara icterocephala*), a resident bird of Costa Rica, and a range of neo-tropical migrants and resident birds in the Eastern USA and the Caribbean, respectively (see full list of hosts in Table 2). However, blood smears either were unavailable or were not examined, and therefore, the sequences deposited under accession numbers AF465555 and JN819334 are likely *P. homopolare* but this was not confirmed microscopically.

Phylogenetic analysis

The final alignment included 20 taxa, each with sequences of 468 nucleotides of the cyt*b* gene. The phylogenetic reconstruction (Fig. 1), as well as morphological evidence (see "Parasite description" section below, and Fig. 2), confirms that *P. homopolare* is in the *Plasmodium* subgenus *Novyella*. The Bayesian phylogenetic tree shown in Fig. 1 includes seven additional species within the subgenus *Novyella*: *Plasmodium homonucleophilum*, *Plasmodium vaughani*, *Plasmodium parahexamerium*, *Plasmodium globularis*, *Plasmodium rouxi*, *Plasmodium multivacuolaris*, and *Plasmodium ashfordi*, as well as one species from the subgenus *Bennettinia*, *Plasmodium juxtanucleare*, which produces small *Novyella*-like erythrocytic meronts. Genetic distance analysis indicates that *P. homopolare* is most closely related to *P. parahexamerium*, with a genetic divergence of 3.6 % (Table 3).

Parasite description

P. (*Novyella*) *homopolare* sp. nov.

Trophozoites Trophozoites (Fig. 2a–d) are seen in mature erythrocytes. The earliest forms can be found anywhere in the host cells but are more often seen in a position subpolar or polar to the nuclei of erythrocytes. Advanced trophozoites are seen only in positions subpolar or polar to the nuclei of erythrocytes, are variable in shape, usually markedly amoeboid in outline, and often possess one or several long outgrowths (Fig. 2c, d). The nuclei and cytoplasm are prominent in advanced trophozoites (Fig. 2c). Each fully grown trophozoite usually possesses a prominent diffuse nucleus, readily visible cytoplasm, one globule (which may be invisible in some parasites), and two or three small, roundish brown pigment granules (Fig. 2b), which are usually located close to each other near the edge of the parasite and may be clumped together (Fig. 2d). The cytoplasm contains one or several small vacuoles. Trophozoites do not adhere to the nuclei of erythrocytes. The "ring" stage is not seen. One tiny bluish refractive globule appears in advanced trophozoites (Fig. 2c, d) but is not visible in all parasites (Fig. 2b). The influence of trophozoites on the morphology of infected erythrocytes is not pronounced.

Erythrocytic meronts Erythrocytic meronts (Fig. 2e–l) are seen mainly in mature erythrocytes, but also occasionally in nearly mature erythrocytes. The cytoplasm is prominent and

Table 1 Geographic and host distribution of *Plasmodium* (*Novyella*) *homopolare* (lineage SOSP CA 3P) reported by PCR-based diagnostics or microscopy

Bird family and species	Locality (co-ordinates)	Accession number
Emberizidae		
Atlapetes albinucha[a]	Colombia (04° 42′ N, 75° 29′ W)	KF537280; KF537287
Melospiza lincolnii	California (36° 44′ N, 119° 29′ W)	KJ482708
Melospiza melodia[a]	California (36° 44′ N, 119° 29′ W)	KJ482708
Passerella iliaca	California (36° 44′ N, 119° 29′ W)	KJ482708
Pipilo maculatus[a]	California (36° 44′ N, 119° 29′ W)	KJ482708
Zonotrichia capensis[a]	Colombia (04° 42′ N, 75° 32′ W; 04°42′ N, 75° 29′ W; 04° 43′ N, 75°27′ W)	KF537291; KF537286; KF537281; KF537289
Icteridae		
Molothrus ater	California (36° 44′ N, 119° 29′ W)	KJ482708
Parulidae		
Myiothlypis coronatus[a]	Colombia (04° 42′ N, 75° 32′ W; 04° 42′ N, 75° 29′ W)	KF537278; KF537288; KF537294
Geothlypis trichas[a]	California (36° 44′ N, 119° 29′ W)	KJ482708
Myioborus ornatus[a]	Colombia (04° 42′ N, 75° 29′ W)	n/a
Cardellina pusilla	California (36° 44′ N, 119° 29′ W)	KJ482708
Sittidae		
Sitta carolinensis	California (36° 44′ N, 119° 29′ W)	KJ482708
Troglodydidae		
Troglodytes aedon	California (36° 44′ N, 119° 29′ W)	KJ482708
Thraupidae		
Anisognathus somptuosus[a]	Colombia (04° 42′ N, 75° 29′ W)	KF537289
Chlorospingus flavigularis[a]	Colombia (04° 42′ N, 75° 32′ W)	n/a

Note that although the accession numbers differ among the Colombian hosts, they represent identical sequences of *Plasmodium homopolare*. Neotropical migrants are indicated in bold font

All reports, except those indicated "n/a" under the Accession number column, are based on PCR-based diagnostics

[a] Denotes hosts in which parasites were seen in blood films, as well as reported by PCR-based diagnostics

stains pale blue in early meronts (Fig. 2e–g). It gradually becomes poorly visible or even invisible in fully grown meronts (Fig. 2h–k). Nuclei are markedly variable in size and form in growing meronts but generally decrease in size as the parasite matures (compare Fig. 2e, f with Fig. 2i, j). Vacuoles are visible in some growing meronts (Fig. 2h). One small bluish refractive globule is often seen in growing meronts (Fig. 2h) and is more often seen in binuclear parasites. However, the globule is not visible in all meronts and gradually degrades in mature meronts to a point where it is often only seen as a bluish rim close to pigment granules (Fig. 2i). The globule is invisible in the majority of mature meronts (Fig. 2, j–l). Occasionally, two small refractive globules are seen in young meronts (Fig. 2h). Growing meronts are markedly variable in shape (elongate, oval, and roundish forms present) and usually possess one or several long amoeboid outgrowths (Fig. 2f, g). Fully grown meronts are usually roundish or slightly oval (Fig. 2j, k) and sometimes fan-like in shape (Fig. 2i). Nuclei are usually arranged irregularly in mature meronts, which produce between four and eight merozoites. The majority of mature meronts (>80 % in all type preparations) possess six and eight merozoites, but meronts with six merozoites predominate. Meronts contain two to four minute brown pigment granules, which, in mature meronts, are usually clumped together and look like a solid mass (Fig. 2j–l). Both growing and mature meronts are strictly of polar or subpolar position to the nuclei of erythrocytes (Fig. 2e–k) and do not adhere to the nuclei; these characteristics appear to be the most distinctive features of this parasite species' development. Mature merozoites do not exceed 1 μm in diameter; their cytoplasm usually is poorly visible or invisible (Fig. 2l). Merozoites persist in infected erythrocytes for some time after the rupture of meronts (Fig. 2l). The influence of meronts on the morphology of infected erythrocytes usually is not pronounced, but nuclei of some infected erythrocytes may be slightly displaced laterally (Fig. 2j, l; Table 4).

Macrogametocytes Macrogametocytes (Fig. 2m–r) develop in mature erythrocytes; the cytoplasm is heterogeneous in appearance, often containing oval bluish globular-like inclusions (Fig. 2q). Gametocytes are elongated in form and slightly irregular in outline from the earliest stages of their development. Growing gametocytes occur lateral to, but never touch, the nuclei of infected erythrocytes and usually adhere to the erythrocytic envelope and extend along the nuclei (Fig. 2m), yet another characteristic feature that is observed in the parasite's development. As the parasite matures, the central part of the gametocyte adheres to the nucleus, leaving its ends unattached (Fig. 2n–q). Mature gametocytes usually adhere to both the nuclei and erythrocytic envelope of

Table 2 Geographic and host distribution of *Plasmodium* spp. with 100 % or nearly 100 % (≤2-nucleotide difference) sequence matches to *Plasmodium homopolare* (lineage SOSP CA 3P), but lacking examination of blood smears

Bird family and species	Geographical region
Cardinalidae	
Passerina ciris	Yucatan Peninsula
Emberizidae	
Arremonops chloronotus	Yucatan Peninsula; Quintana Roo, Mexico
Melospiza melodia	Chicago, Illinois
Fringillidae	
Carduelis tristis	Chicago, Illinois
Icteridae	
Icterus leucopteryx	Jamaica
Mimidae	
Dumetella carolinensis	Chicago, Illinois
Paridae	
Baeolophus bicolor	Ozark Mountains, Missouri
Parulidae	
Setophaga caerulescens	Jamaica
Setophaga dominica	Ozark Mountains, Missouri
Setophaga magnolia	Ozark Mountains, Missouri
Geothlypis trichas	Chicago, Illinois; Kalamazoo, Michigan; Yucatan Peninsula; Kellogg Biological Station, Michigan; St. Louis Missouri
Helmitheros vermivorus	Ozark Mountains, Missouri; Jamaica
Mniotilta varia	Ozark Mountains, Missouri; Jamaica
Geothlypis formosa	Ozark Mountains, Missouri
Setophaga americana	Ozark Mountains, Missouri; Jamaica
Seiurus aurocapillus	Chicago, Illinois; Ozark Mountains, Missouri
Setophaga citrina	Ozark Mountains, Missouri
Thraupidae	
Coereba flaveola	Dominican Republic, Jamaica
Tangara icterocephala	Costa Rica
Euneornis campestris	Jamaica

Neotropical migrants are indicated in bold font

erythrocytes, filling-up the poles of erythrocytes and slightly enclosing the erythrocyte nuclei with their ends, but not encircling them completely (Fig. 2r). The macrogametocyte nucleus appears relatively diffuse and irregular in shape and has faint boundaries and a central or subcentral position. Clumps of chromatin are very visible within the parasite nucleus (Fig. 2o). Small- (<0.5 μm) and medium- (0.5–1.0 μm) sized roundish and oval pigment granules can be seen anywhere within the gametocyte (Fig. 2p, q). The influence of gametocytes on the morphology of infected erythrocytes is usually not pronounced, but nuclei of some infected erythrocytes are slightly displaced laterally (Table 4).

Microgametocytes The general configuration and other features are similar to macrogametocytes (Fig. 2s, t), with the usual haemosporidian sexual dimorphic characters; however, the granular appearance of the cytoplasm is less evident than that in macrogametocytes. The nucleus is markedly diffuse to the point where its size cannot be easily measured.

Remarks

Because of its small erythrocytic meronts and elongated microgametocytes, *P. homopolare* belongs to the subgenus *Novyella* (Corradetti et al. 1963, Garnham 1966). This malaria parasite can be readily distinguished from other species of *Novyella* primarily due to its strictly polar or subpolar position of meronts and advanced trophozoites in infected erythrocytes (Fig. 2a–k). The positioning of the meronts and advanced trophozoites is shared with the relatively rare avian parasite *Plasmodium polare* (Manwell 1935; Bishop and Bennett 1992), which is reflected in the name of this new species: *P. homopolare*. *P. polare* produces meronts of greater size than *P. homopolare*, and, in this regard, is closer to species of the subgenus *Giovannolaia* (Valkiūnas 2005). The maximum number of merozoites in mature meronts of *P. homopolare* is 8, as opposed to 16 in those of *P. polare*, allowing for differentiation of these two species.

The morphology of blood stages of *P. homopolare* was strictly consistent across the eight California and Colombia bird species represented in the Emberizidae, Parulidae, and Thraupidae families (Fig. 2), indicating that the parasite did not change main morphological characters in different avian hosts belonging to Passeriformes. Morphometric comparisons of mature meronts, macrogametocytes, and microgametocytes of *P. homopolare* in the Song sparrow (*Melospiza melodia*) and the Rufous-collared sparrow (*Z. capensis*) sampled in California and Colombia, respectively, did not differ significantly ($P \geq 0.4$, for all features, except the gametocyte area, for which $P = 0.09$, data not shown). Due to insufficient intensity of parasitemia with some blood stages (usually mature gametocytes and mature meronts), we were unable to complete a similar morphometric analysis for the other bird species in our study. Morphometric characters of parasites in the type vertebrate host (*Melospiza melodia*) are given in Table 4.

Taxonomic summary

Type host: Song sparrow *Melospiza melodia* (Passeriformes, Emberizidae).

DNA sequences: Mitochondrial cyt*b* lineage SOSP CA 3P (478 bp, GenBank accession no. KJ482708).

Additional hosts: Numerous species of passeriform birds, belonging mainly to Emberizidae (Table 1).

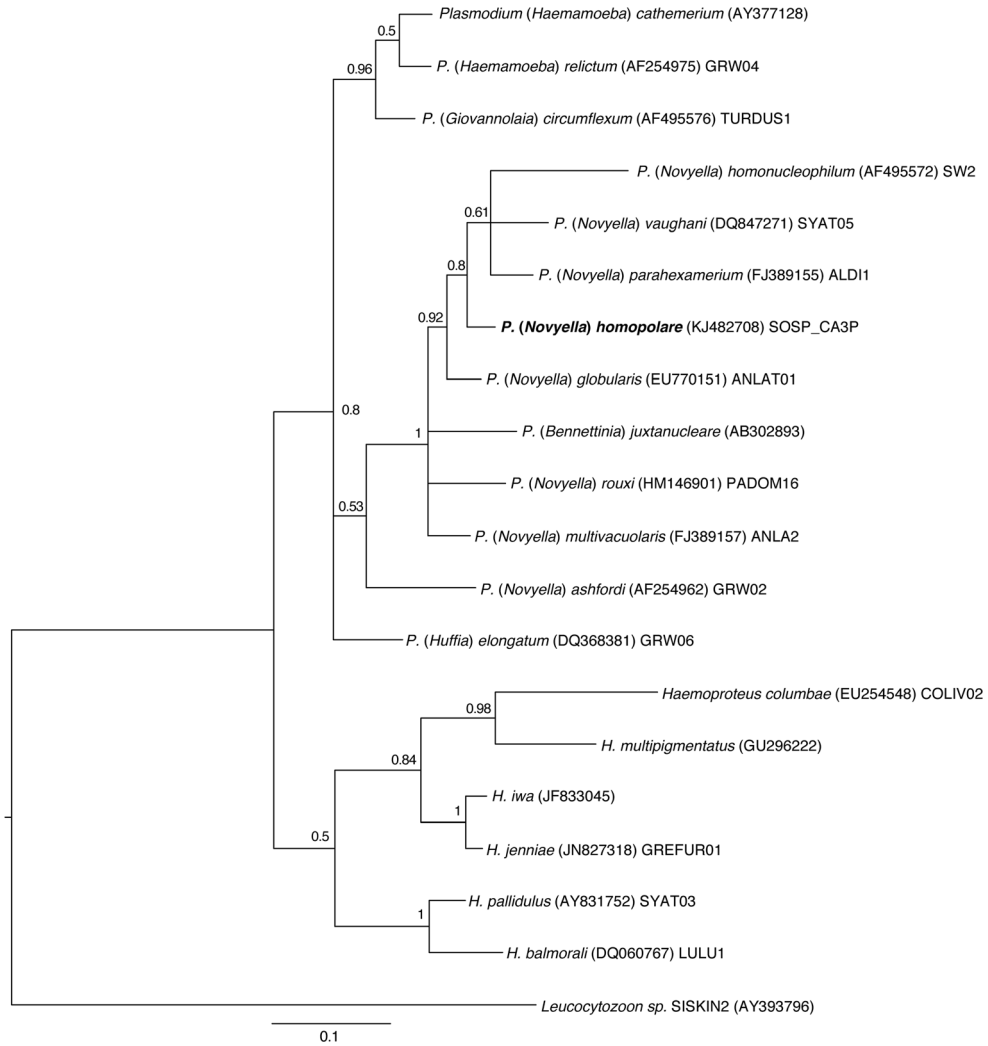


Fig. 1 Bayesian inference phylogenetic reconstruction based on 468 nucleotides of the cytochrome *b* gene and including *Plasmodium* (*Novyella*) *homopolare*, as well as 19 reference sequences of known morphospecies from GenBank. *Leucocytozoon* sp. SISKIN2 (accession no. AY393796) serves as the out-group. Note that *P. homopolare* groups with *Plasmodium* species that are primarily in the subgenus *Novyella*

Type locality: China Creek County Park, Fresno County, California, USA (36° 44′ N, 119° 29′ W, 120 m above sea level).

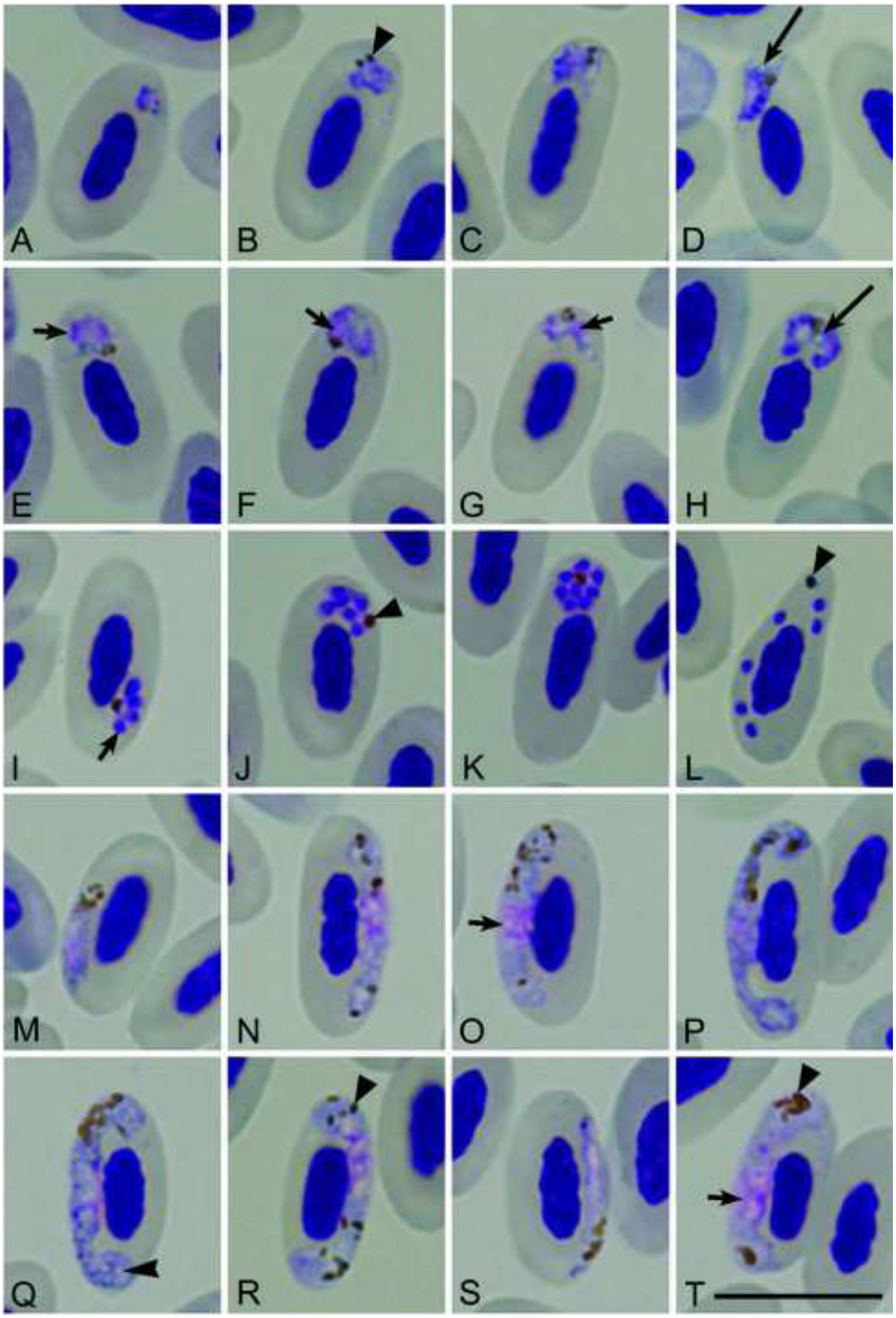
Prevalence: The prevalence determined using combined PCR-based detection and microscopy methods was 28 of 51 (54.9 %) in *Melospiza melodia* at the type locality. Overall PCR-based prevalence in all investigated birds was 21 and 3.6 % in California and Colombia, respectively. However, in California, all bird blood samples were analyzed using PCR whereas, in Colombia, only those birds diagnosed by microscopy were analyzed using PCR.

Geographical distribution: *P. homopolare* and its lineages have been recorded only in North and South America where the parasite is present in numerous species of migrating and resident birds. *P. homopolare* is a host generalist (Table 1), but its transmission has not been reported outside of the American continents.

Site of infection: Mature or nearly mature erythrocytes; no other data.

Type specimens: Hapantotype (accession no. 48740 NS, lineage SOSP CA 3P, intensity of parasitemia is approximately 8 %, *Melospiza melodia*, China Creek County Park, Fresno County, California, 36° 44′ N, 119° 29′ W, 21 January 2013, E. Walther) is deposited in the Nature Research Centre, Vilnius, Lithuania. Parahapantotypes are deposited in the Queensland Museum, Queensland, Australia (accession no. G465687, other data as for the hapantotype) and in the Nature Research Centre (accession nos. 48741, 48742 NS, lineage SOSP CA 3P, host is *Pipilo maculatus*, China Creek County Park, Fresno County, CA, 24 June 2011, collected by E. Walther).

Additional material: Blood films from *Pipilo maculatus* (accession nos. 48744–48747 NS, 48751–48754 NS), *Melospiza melodia* (48748–48750 NS), and *Geothlypis trichas* (48755 NS) were deposited in the Nature Research Centre, Vilnius, Lithuania. Blood films from *Myiothlypis coronatus* (accession nos. GERPH-07316, GERPH-07317, and GERPH-07318) and *Z. capensis* (accession nos. GERPH-04822, GERPH-04823, and GERPH-04824) were deposited in the collection Grupo de Estudio Relación Parásito Hospedero (GERPH), Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia.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◀ **Fig. 2** *Plasmodium* (*Novyella*) *homopolare* sp. nov. (lineage SOSP CA 3P) from the blood of Song sparrow *Melospiza melodia*. **a**–**d** Trophozoites. **e**–**l** Erythrocytic meronts. **m**–**r** Macrogametocytes. **s**, **t** Microgametocytes. *Short arrows* nuclei of parasites, *long arrows* bluish refractive globule, *triangle arrowhead* pigment granules, s*imple arrowhead* globular-like inclusions in the cytoplasm. Giemsa-stained thin blood films. *Scale bar* = 10 μm

Digital images of blood stages of the new parasite are available on request from GERPH.

Etymology: The species name reflects the similarity of erythrocytic meronts and advanced trophozoites of this parasite to *P. polare*. In both parasites, these blood stages assume a strictly polar or subpolar position in infected erythrocytes, a characteristic feature of their development.

Discussion

P. homopolare was identified to morphospecies based on blood samples from neo-tropical migrants and resident birds in California, USA, and Colombia. We believe *P. homopolare* is present in birds throughout the New World because the California and Colombia cyt*b* sequences matched 100 % or nearly 100 % (i.e., ≤2-nucleotide difference) to two previously deposited parasite sequences in GenBank: accession nos. AF465555 and JN819334. These sequences were isolated from the Silver-throated tanager (*T. icterocephala*), a resident bird of Costa Rica, and a variety of Neotropical migrants and resident birds in the Eastern USA and the Caribbean, respectively (see full list of hosts in Table 2). Because blood smears either were unavailable or were not examined for these hosts, we cannot unequivocally confirm AF465555 and JN819334 to be *P. homopolare*; it is likely based on the available data (Table 1) but would require additional testing because genetic differences among cyt*b* gene partial sequences in some readily distinguishable haemosporidian species is small (Hellgren et al. 2007). Additionally, it should be noted that the development of *Plasmodium* spp. and related haemosporidians can be abortive in resistant or partly resistant hosts, resulting in no development of gametocytes (Valkiūnas et al. 2011; Cannell et al. 2013). This can happen when (1) sporozoites are inoculated by a vector into the blood but are incapable of intracellular invasion, so remain in the circulation for some time, or (2) remnants of tissue meronts, the development of which was aborted before the gametocyte stage, are produced and remain in hosts, serving as a PCR template. In both cases, the bird would be scored as infected by PCR when, in fact, it might not be a competent host of the parasite. Therefore, conclusions regarding the full range of avian hosts would require microscopic examination of blood films in addition to comparison of molecular results.

Assuming that *P. homopolare* is broadly distributed throughout the New World and infects a wide range of hosts in geographic areas where haemosporidian studies have occurred, it is notable that this parasite has not previously been identified in prior studies from North America and the Neotropics, particularly those conducted in California and Colombia.

Previous haemosporidian studies in the Nearctic

Numerous studies examining the prevalence and diversity of *Plasmodium* spp. have been conducted in the Nearctic since the 1930s (Herman 1938; Hewitt 1940; Herman et al. 1954; Clark and Swinehart 1966; Greiner et al. 1975; Super and van Riper 1995; Ricklefs et al. 2005; Martinsen et al. 2008). In particular, a 6-year study by Herman et al. (1954) was undertaken in Kern County, California, approximately 160 km from the California study site at China Creek County Park. In Kern County, numerous species of *Plasmodium* were identified morphologically, including *Plasmodium elongatum*, *Plasmodium hexamerium*, *Plasmodium nucleophilum*, *P. polare*, *Plasmodium relictum*, *P. rouxi*, and *P. vaughani*, as well as several unknown species from 7,440 passerine and

Table 3 Genetic distances between *Plasmodium homopolare* and seven other *Plasmodium* spp. of the subgenus *Novyella*, as well as an out-group (*P. elongatum*)

	Accession no.	Species (lineage)	1	2	3	4	5	6	7	8	9
1	KJ482708	*P. homopolare* (SOSP CA 3P)	–								
2	FJ389155	*P. parahexamerium* (ALDI1)	3.6	–							
3	EU770151	*P. globularis* (ANLA1)	3.8	4.1	–						
4	DQ847271	*P. vaughani* (SYAT05)	4.7	4.1	4.7	–					
5	HM146901	*P. rouxi* (PADOM16)	4.7	5.8	5.6	5.6	–				
6	FJ389157	*P. multivacuolaris* (ANLA2)	5.6	5.1	4.7	6.2	5.4	–			
7	AF495572	*P. homonucleophilum* (SW2)	6.6	6.2	7.5	6.4	7.3	8.8	–		
8	AF254962	*P. ashfordi* (GRW02)	6.8	8.1	7.5	7.9	7.5	8.3	7.9	–	
9	DQ368381	*P. elongatum* (GRW06)	7.9	8.1	8.3	7.9	8.6	8.1	9.4	6.8	–

Genetic divergence is given as a percentage and was calculated using uncorrected *p* distance analysis, based on 468 nucleotides of the cyt*b* gene

Table 4 Morphometry of mature erythrocytic meronts, gametocytes, and host-cells of *Plasmodium homopolare* (lineage SOSP CA 3P) from the blood of the Song sparrow *Melospiza melodia*

Feature	Measurements[a]
Uninfected erythrocyte	
Length	10.3–12.8 (11.4 ± 0.5)
Width	5.2–6.8 (6.0 ± 0.3)
Area	49.7–64.8 (55.2 ± 4.2)
Uninfected erythrocyte nucleus	
Length	4.8–6.3 (5.3 ± 0.3)
Width	2.4–3.1 (2.5 ± 0.2)
Area	9.5–19.4 (12.2 ± 1.3)
Meront	
Length	3.2–6.0 (4.3 ± 0.3)
Width	1.9–3.6 (2.5 ± 0.5)
Area	6.1–15.2 (8.4 ± 1.4)
No. of merozoites	4–8 (6.9 ± 0.7)
Infected erythrocyte	
Length	10.1–13.8 (11.8 ± 0.7)
Width	5.0–6.4 (5.6 ± 0.4)
Area	47.4–62.8 (52.7 ± 4.6)
Infected erythrocyte nucleus	
Length	4.4–6.6 (5.4 ± 0.4)
Width	2.1–3.2 (2.4 ± 0.3)
Area	9.3–18.8 (12.7 ± 1.6)
Macrogametocyte	
Length	10.7–16.7 (13.8 ± 1.5)
Width	1.3–3.0 (2.1 ± 0.4)
Area	18.9–34.0 (27.0 ± 3.7)
Gametocyte nucleus	
Length	1.6–5.5 (2.4 ± 0.8)
Width	0.9–2.3 (1.5 ± 0.3)
Area	1.3–5.4 (3.2 ± 2.0)
Pigment granules	5–15 (9.5 ± 1.8)
NDR	0.6–1.1 (0.9 ± 0.1)
Infected erythrocyte	
Length	10.5–13.8 (11.6 ± 0.9)
Width	5.0–7.2 (6.2 ± 0.5)
Area	49.0–68.9 (58.2 ± 4.9)
Infected erythrocyte nucleus	
Length	4.5–6.0 (5.0 ± 0.3)
Width	2.2–3.0 (2.4 ± 0.2)
Area	9.3–17.4 (11.2 ± 1.0)
Microgametocyte	
Length	10.2–18.9 (14.2 ± 1.5)
Width	1.4–3.0 (2.4 ± 0.5)
Area	22.2–36.7 (28.8 ± 3.8)
Gametocyte nucleus	
Length	_[b]
Width	_[b]
Area	_[b]
Pigment granules	7–17 (10.3 ± 2.6)
NDR	0.5–1.1 (0.8 ± 0.2)
Infected erythrocyte	
Length	10.3–13.8 (11.7 ± 0.9)
Width	5.2–7.0 (6.2 ± 0.4)
Area	47.9–69.8 (58.2 ± 5.0)
Infected erythrocyte nucleus	
Length	4.4–6.3 (5.3 ± 0.4)
Width	2.0–3.1 (2.5 ± 0.3)
Area	9.2–18.4 (12.0 ± 1.5)

NDR nucleus displacement ratio according to Bennett and Campbell (1972)

[a] All measurements (n = 21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation

[b] Difficult to measure feature; see the text for explanation

near-passerine blood smears. *P. relictum* was the most common parasite, infecting 792 (10.7 %) birds. In Kern County, the vast majority of birds sampled were House sparrows (*Passer domesticus*) (2,880/7,440) and House finches (*Carpodacus mexicanus*) (1,741/7,440). Two hundred forty-three (8.1 %) House sparrows and 424 (24.4 %) House finches were infected with *P. relictum*. At China Creek, we collected samples from six House finches, none of which was infected with *P. relictum*. We sampled no House sparrows. Only one isolate of *P. relictum* was obtained at China Creek and that was from a Cliff swallow (*Petrochelidon pyrrhonota*) (data not shown). In the Kern County study, blood smears were also obtained from the two host species at China Creek found to have high rates of infection with *P. homopolare*: Song sparrows (*Melospiza melodia*) and Spotted towhees (*Pipilo maculatus*). Herman et al. (1954) trapped 14 Song sparrows, of which 2 (14.3 %) were infected with *P. relictum*, and 42 Spotted towhees, of which 4 (9.5 %) were infected with *P. relictum*, 1 (2.4 %) with *P. hexamerium*, and 1 (2.4 %) with *P. vaughani*. None of the parasites in these infected birds was categorized as unknown *Plasmodium* species, as would be expected if the authors had found the then undescribed *P. homopolare* in the hosts. Conversely, none of the six hosts in which the authors identified an unknown *Plasmodium* species was host that harbored *P. homopolare* in the China Creek study.

Additional studies in the Nearctic utilizing microscopy identified several *Plasmodium* spp., including *Plasmodium praecox*, *Plasmodium circumflexum*, and *P. relictum* in California (Herms et al. 1939; Super and van Riper 1995; Martinsen et al. 2008) and *Plasmodium cathemerium*, *P. praecox*, *P. circumflexum*, *P. vaughani*, *P. elongatum*,

P. nucleophilum, and *P. hexamerium* in Cape Cod, Massachusetts (Herman 1938). Note that the name *P. praecox* is now invalid and the parasite observed was likely *P. relictum* (Valkiūnas 2005). *P. relictum* and *P. cathemerium* have also been identified in songbirds obtained from Mexican markets (Hewitt 1940).

A few of the avian malaria studies referenced above also provided descriptions of *Plasmodium* spp. that were not recognized by the authors as named morphospecies. Herman et al. (1938) reported that the majority of undetermined species had elongate gametocytes and probably belonged to either *P. vaughani*, *P. nucleophilum*, or *P. hexamerium* or a species with similar morphology, but not to *P. polare*, the parasite most similar to *P. homopolare*. Herman (1938) also observed that more than four merozoites were observed in at least one erythrocytic meront (schizont). Hewitt (1940) compared an unknown *Plasmodium* spp. in his study to *P. nucleophilum* and described it as a small parasite "generally localized at the ends of the host cell near the nucleus, many times in direct contact with the nucleus. The number of merozoites in what seemed to be mature segmenters averaged from 5 to 7. No gametocytes were found." Some studies also reported at least some portion of *Plasmodium* infections identified in their studies to the genus level only (Herman et al. 1954; Clark and Swinehart 1966; Martinsen et al. 2008), generally due to low parasitemia.

Previous haemosporidian studies in the Neotropics

Numerous studies of avian hematozoa in Colombia have been conducted since at least 1952. In all cases, *Plasmodium* infections were detected using blood smears and their microscopic examination, most parasites were identified to genus or subgenus only, and *Plasmodium* prevalence was generally low, <5 % (Bennett and Borrero 1976; Rodriguez and Matta 2001; Valkiūnas et al. 2003; Basto et al. 2006). *Plasmodium* species identified in previous studies in Colombia include *Plasmodium lutzi*, *Plasmodium unalis*, and, tentatively, *P. relictum* (Matta et al. 2004; Mantilla et al. 2013a, b). Several studies have also been published on blood parasites in Panamanian birds in which microscopy was used to identify *Plasmodium* morphospecies, including *P. relictum*, *P. cathemerium*, *P. polare*, *P. hexamerium*, *P. vaughani*, *P. circumflexum*, *P. nucleophilum*, *P. pinotii*, and *P. rouxi* (Galindo and Sousa 1966; Sousa and Herman 1982), though the authors acknowledged that low parasitemia and limited sampling may have resulted in erroneous identifications (Sousa and Herman 1982). In a Monteverde, Costa Rica study utilizing microscopy, *P. relictum* was found in low prevalence (Young et al. 1993). In Jamaica, avian sampling revealed infections of *P. relictum*, *P. vaughani*, and *P. pinotii* (Bennett et al. 1980).

Some studies in Colombia, Costa Rica, and Jamaica reported at least some portion of *Plasmodium* infections identified in their studies to the genus or subgenus level, particularly the subgenera *Novyella* and *Haemamoeba* (Bennett and Borrero 1976; Bennett et al. 1980; Rodríguez and Matta 2001; Valkiūnas et al. 2004; Basto et al. 2006; Rodríguez et al. 2009).

The lack of identification of *P. homopolare* in previous studies, particularly the Herman et al. (1954) study in California, with its relatively high prevalence of *Plasmodium* spp., begs the question: Why has *P. homopolare* not been described previously? *P. homopolare* may have been misidentified as some other *Plasmodium* (*Novyella*) spp. or was impossible to describe due to predominant light parasitemias, absence of all blood stages necessary for parasite identification, and frequent co-infections. All these cases are common in naturally infected birds and are obstacles in parasite species identification and, particularly, new species descriptions.

Possible previous misidentification of *P. homopolare*

Herman et al. (1954) identified *P. relictum* (subgenus *Haemamoeba*) in *Melospiza melodia*, the type species we have designated for *P. homopolare*. It is unlikely that this represents a misidentification because of multiple morphological differences between *P. homopolare* and *P. relictum* (Valkiūnas 2005). The various erythrocytic life stages of *P. relictum* and other parasites in the subgenus *Haemamoeba* look distinctly different from and affect the host red blood cell differently than those of the subgenus *Novyella*. However, *P. homopolare* could have been misidentified as *P. hexamerium* or *P. vaughani*, which Herman et al. (1954) isolated from Spotted towhees. *P. hexamerium* and *P. vaughani*, which are in the subgenus *Novyella*, share a number of morphological features with *P. homopolare*: (1) trophozoites can be amoeboid in outline, (2) mature erythrocytic meronts often contain six merozoites, (3) pigment granules are often aggregated into a solid dark mass (in *P. homopolare* and *P. hexamerium* only), and (4) gametocytes are elongate and have a variable outline (more common in *P. vaughani* and *P. homopolare* than *P. hexamerium*). Meronts of *P. vaughani* and *P. hexamerium* are never strictly polar in position in infected erythrocytes, but this feature might be overlooked due to light parasitemia. While microscopy might not have allowed a distinction to be made between light parasitemia infections of *P. homopolare* and other *Plasmodium* spp. in the same morphologically similar species complex, DNA sequencing would allow such differentiation for the first time.

Several of the Colombian studies presented above included unidentified *Plasmodium Novyella* species, which could have been *P. homopolare*. Examination of preserved slides

deposited in the GERPH collection from some of these studies (Rodríguez and Matta 2001; Matta et al. 2004; Basto et al. 2006; Rodríguez et al. 2009) showed that *Plasmodium* (*Novyella*) species found in Emberizidae, Icteridae, and Thraupidae hosts correspond to *P. unalis* and *P. nucleophilum* (data not shown). Unfortunately, the identity of unknown *Plasmodium* species in the *Novyella* subgenus from Bennett and Borrero (1976) and Valkiūnas et al. (2003) cannot be confirmed since morphological descriptions or slides are not available. Future studies should include an examination of preserved slides from some of these older studies, to determine the possible presence of *P. homopolare*.

Light parasitemias and co-infections in earlier studies prevented description of *P. homopolare*

The disease dynamics of avian haemosporidians include a brief pre-patent period in which the parasites are found only in the host tissues, followed by a patent stage that begins with short acute infection lasting on the order of weeks and characterized by high parasitemia (Valkiūnas 2005). Assuming that the host survives, the acute infection is followed by an indefinite period of chronic infection, which is characterized by low parasitemia (Garnham 1966; Valkiūnas 2005; Atkinson 2008). It is during this chronic infection that researchers are most likely to trap a bird because this period is relatively long and the bird is more likely to be active during the chronic infection than during the acute infection. In fact, the acute stage of *Plasmodium* is relatively short, and the parasitemia level during chronic infections is relatively low, compared to other haemosporidians (Greiner et al. 1975; Valkiūnas 2005). In addition, the patent infection is affected by season; for example, *Plasmodium* spp. are unlikely to be found in hosts in early spring and late fall in the Holarctic region using microscopic examination of blood films (Valkiūnas 2005). Moreover, co-infections of haemosporidian parasites are common in many bird species and confound the morphological identification of these parasites (Valkiūnas et al. 2008a). All of these factors present a challenge to the malaria researcher relying solely on microscopy to quantify prevalence and parasite identity in a population and would necessitate frequent sampling and examination of multiple blood smears to find and identify morphospecies of *Plasmodium* parasites. All of the previous haemosporidian studies described above relied solely on microscopy and sometimes on a single blood smear for each host examined. It is not always clear in which season(s) sampling took place, but it is possible that some early researchers in the Holarctic were unaware of the seasonal dynamics of *Plasmodium* infections and may have collected samples during bird migration in early spring and late fall when parasitemias are light or absent for certain *Plasmodium* species. Many studies were able to identify *Plasmodium* parasites to morphospecies, but most included samples for which there was not enough morphological data to identify the parasites to species.

Phylogenetic relationship of *P. homopolare* to other morphospecies

Phylogenetic analysis placed *P. homopolare* in a clade with seven other species within the subgenus *Novyella*. Parasites of the *Novyella* subgenus are distinguished from other subgenera by the following morphological characteristics: (1) scanty cytoplasm in erythrocytic meronts, (2) size of fully grown erythrocytic meronts does not exceed or only slightly exceeds that of the nuclei of infected erythrocytes, (3) fully grown gametocytes are elongated, and (4) exoerythrocytic merogony takes place in cells of the reticuloendothelial system (Garnham 1966; Valkiūnas 2005). *P. vaughani* is the type species of the *Novyella* subgenus and has a worldwide geographic distribution with the exception of Antarctica. *Novyella* species are known to infect over 240 species of Passeriformes (Valkiūnas 2005; Bensch et al. 2009) including species from the Sylviidae, Turdidae, Fringillidae, Petroicidae, Muscicapidae, Meliphagidae, and Alaudidae families. Genetic distance analysis indicates that *P. homopolare* has the lowest levels of genetic divergence from *P. parahexamerium*. The latter parasite was first identified in 2009 in two species from the Turdidae family, as well as an Old world warbler, *Hylia prasina* (Green hylia), in Africa (Valkiūnas et al. 2009; Bensch et al. 2009). Interestingly, *P. homopolare*, thus far, has only been confirmed to infect hosts in the New World. However, like *P. homopolare*, *P. parahexamerium* was identified relatively recently, and further study may reveal that one or both of these species infect a wider range of hosts on a broader geographical scale than is currently understood.

The application of molecular markers opens new opportunities for *Plasmodium* spp. taxonomic studies due to the possibility of using sequence information to strengthen studies of wild hosts with light parasitemias. Many species of *Plasmodium* occur in birds belonging to different species, genera, families, and even orders. Within this host range, the same morphospecies may exhibit slightly different morphological forms, resulting in strains, varieties, and transmission by different vectors throughout their distributional range. Due to these morphological variants, it has been the convention in early avian malaria studies (Garnham 1966; Atkinson 2008) that any new species should only be established if supported by a "package" of taxonomic data, including the full range of blood forms, and also data on tissue merogony, periodicity, vectors, and other features. It is difficult and time-consuming to collect such information, which resulted in few descriptions of new *Plasmodium* species before DNA sequencing became available (Garnham 1966; Valkiūnas 2005). The combined

application of sequence data and digital microscopy techniques provides new opportunities to repeat studies and use light microscopy for parasite species identification; this strategy has benefitted taxonomic research and has resulted in the current renaissance in haemosporidian parasite taxonomy and descriptions of numerous new haemosporidians parasitizing birds (Valkiūnas et al. 2007; Valkiūnas et al. 2008c; Valkiūnas et al. 2009; Levin et al. 2012; Ilgūnas et al. 2013; Mantilla et al. 2013a; Valkiūnas et al. 2013) and reptiles (Perkins and Austin 2009). Importantly, the descriptions of new morphospecies are in accord with recent PCR-based studies indicating remarkable genetic diversity of haemosporidian parasites (Bensch et al. 2000; Ricklefs and Fallon 2002; Beadell et al. 2004; Ricklefs et al. 2005; Valkiūnas 2005; Loiseau et al. 2010). This study thus reports the identifying molecular and morphological characters for the detection of *P. homopolare*, a widespread malaria parasite of American birds.

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References

Atkinson CT (2008) Chapter 3. Avian malaria. In Parasitic diseases of wild birds, 1st edn. Wiley-Blackwell, Iowa, pp 35–53

Basto N, Rodríguez OA, Marinkelle CJ, Gutierrez R, Matta N (2006) Haemoatozoa in birds from La Macarena National Natural Park (Colombia). Caldasia 28(2):371–377

Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, Atkinson CT, Fleischer RC (2004) Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Mol Ecol 3:3829–3844

Bennett GF, Campbell AG (1972) Avian Haemoproteidae. I. Description of Haemoproteus fallisi n. sp. and a review of the haemoproteids of the family Turdidae. Can J Zool 50:1269–1275

Bennett GF, Borrero JI (1976) Blood parasites of some birds from Colombia. J Wildl Dis 12:454–458

Bennett GF, Whitt H, White H (1980) Blood parasites of some Jamaican birds. J Wildl Dis 16:29–38

Bensch S, Sternman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, Pinheiro RT (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc Lond B Biol Sci 267:1583–1589

Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol 9:1353–1358. doi:10.1111/j.1755-0998.2009.02692.x

Bishop MA, Bennett GF (1992) Host-parasite catalogue of the avian haematozoa: supplement 1 and bibliography of the avian blood-inhabiting haematozoa: supplement 2. Mem Univ Newfoundl Occas Pap Biol 15:1–244

Cannell BL, Krasnec KV, Campbell K, Jones HI, Miller RD, Stephens N (2013) The pathology and pathogenicity of a novel *Haemoproteus* spp. infection in wild Little Penguins (*Eudyptula minor*). Vet Parasitol 197(1-2):74–84. doi:10.1016/j.vetpar.2013.04.025

Clark GW, Swinehart B (1966) Blood protozoa of passerine birds of the Sacramento (Calif.) region. Bull Wildl Dis Assoc 2:53–54

Corradetti A, Garnham PCC, Laird M (1963) New classification of the avian malaria parasites. Parassitologia 5:1–4

Galindo P, Sousa O (1966) Blood parasites of birds from Almirante, Panama, with ecological notes on the hosts. Rev Biol 14(1):27–46

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci: CABIOS 12(6):543–548

Garnham PCC (1966) Malaria parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford

Greiner EC, Bennett GF, White EM, Coombs RF (1975) Distribution of the avian hematozoa of North America. Can J Zool 53:1762–1787

Hellgren O, Waldenström J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol 90:797–802

Hellgren O, Križanauskienė A, Valkiūnas G, Bensch S (2007) Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). J Parasitol 93:889–896

Herman CM (1938) The relative incidence of blood protozoa in some birds from Cape Cod. Tr Am Micr Soc 47:132–141

Herman CM, Reeves WC, McClure HE, French EM, Hammon W (1954) Studies on avian malaria in vectors and hosts of encephalitis in Kern County, CA: infections in avian hosts. Am J Trop Med Hyg 3:676–695

Herms WB, Kadner CG, Galindo P, Armstrong DF (1939) Blood parasites of California birds. J Parasitol 25(6):511–512

Hewitt R (1940) Studies on blood protozoa obtained from Mexican wild birds. J Parasitol 26(4):287–295

Ilgūnas M, Palinauskas V, Iezhova TA, Valkiūnas G (2013) Molecular and morphological characterization of two avian malaria parasites (Haemosporida: Plasmodiidae), with description of *Plasmodium homonucleophilum* n. sp. Zootaxa 3666(1):49–61

Levin II, Valkiūnas G, Iezhova TA, O'Brien SL, Parker PG (2012) Novel *Haemoproteus* species (Haemosporida: Haemoproteidae) from the Swallow-tailed gull (*Lariidae*), with remarks on the host range of Hippoboscid-transmitted avian hemoproteids. J Parasitol 98(4):847–854

Loiseau C, Iezhova T, Valkiūnas G, Chaser A, Hutchinson W, Buermann T, Smith TB, Sehgal RNM (2010) Spatial variation of hemosporidian parasite infection in African rainforest bird species. J Parasitol 96:21–29

Mantilla JS, González AD, Valkiūnas G, Moncada LI, Matta NE (2013a) Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland Colombia. Parasitol Res 112:4193–4204

Mantilla JS, Matta NE, Pacheco MA, Escalante AA, González AD, Moncada LI (2013b) Identification of *Plasmodium* (*Haemamoeba*) *lutzi* (Lucena, 1939) from *Turdus fuscater* (Great Thrush) in Colombia. J Parasitol 99(4):662–668

Manwell RD (1935) How many species of avian malaria parasites are there? Am J Trop Med 15(3):265–283

Martinsen ES, Blumberg BJ, Eisen RJ, Schall JJ (2008) Avian hemosporidian parasites from northern California oak woodland and chaparral habitats. J Wildl Dis 44(2):260–268

Matta NE, Basto N, Gutierrez R, Rodríguez OA, Greiner EC (2004) Prevalence of blood parasites in Tyrannidae (flycatchers) in the eastern plains of Colombia. Mem Inst Oswaldo 99(3):271–274

Muñoz E, Ferrer D, Molina RRA (1999) Prevalence of haematozoa in birds of prey in Catalonia, northeast Spain. Vet Rec 144:623–636

Nylander JAA, Ronquist JP, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. Syst Biol 53:47–67

Perkins SL, Austin CC (2009) Four new species of *Plasmodium* from New Guinea lizards: integrating morphology and molecules. J Parasitol 95:424–433

Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites. Proc R Soc London 269:885–892

Ricklefs RE, Swanson BL, Fallon SM, Martinez-Abrain A, Scheuerlein A, Gray J, Latta SC (2005) Community relationships of avian malaria parasites in Southern Missouri. Ecol Monogr 75(4):543–559

Rodríguez OA, Matta NE (2001) Blood parasites in some birds from eastern plains of Colombia. Mem Inst Oswaldo Cruz 96:000–000

Rodríguez OA, Moya H, Matta NE (2009) Avian blood parasites in the National Natural Park Chingaza: high Andes of Colombia. Hornero 24(1):1–6

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12):1572–1574. doi:10.1093/bioinformatics/btg180

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Harbour Laboratory Press, New York, 2222

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. doi:10.1038/nmeth.2089

Sehgal RNM, Lovette IJ (2003) Molecular evolution of three avian neurotrophin genes: implications for proregion functional constraints. J Mol Evol 57:335–342

Sehgal RNM, Hull AC, Anderson NL, Valkiūnas G, Markovets MJ, Kawamura S, Tell LA (2006) Evidence for cryptic speciation of *Leucocytozoon* spp. (Haemosporida, Leucocytozoidae) in diurnal raptors. J Parasitol 92(2):375–379

Sousa OE, Herman CM (1982) Blood parasites of birds from Chiriqui and Panama provinces in the Republic of Panama. J Wildl Dis 16(2):205–221

Super PE, van Riper C (1995) A comparison of avian hematozoan epizootiology in two California coastal scrub communities. J Wildl Dis 31(4):447–461

Swofford D (2001) *PAUP** 4.0. Sinauer Associates

Valkiūnas G, Salaman P, Iezhova TA (2003) Paucity of Hematozoa in Colombian birds. J Wildl Dis 39(2):445–448

Valkiūnas G, Iezhova TA, Brooks DR, Hanelt B, Brant SV, Sutherlin ME, Causey D (2004) Additional observations on blood parasites of birds in Costa Rica. J Wildl Dis 40(3):555–561

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton

Valkiūnas G, Zehtindjiev P, Hellgren O, Ilieva M, Iezhova TA, Bensch S (2007) Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium* (*Novyella*) *ashfordi* sp. nov. Parasitol Res 100:1311–1322

Valkiūnas G, Iezhova TA, Krizankauskiene A, Palinauskas V, Sehgal RN, Bensch S (2008a) A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J Parasitol 94(6):1395–1401. doi:10.1645/GE-1570.1

Valkiūnas G, Atkinson CT, Bensch S, Sehgal RNM, Ricklefs R (2008b) Parasite identifications in GenBank: how to minimize their number? Trends Parasitol 24(6):247–248

Valkiūnas G, Iezhova TA, Loiseau C, Chasar A, Smith TB, Sehgal RNM (2008c) New species of haemosporidian parasites (Haemosporida) from African rainforest birds, with remarks on their classification. Parasitol Res 103:1213–1228

Valkiūnas G, Iezhova TA, Loiseau C, Smith TB, Sehgal RNM (2009) New malaria parasites of the subgenus *Novyella* in African rainforest birds, with remarks on their high prevalence, classification and diagnostics. Parasitol Res 104:1061–1077

Valkiūnas G, Sehgal RNM, Iezhova TA, Hull AC (2010) Identification of *Leucocytozoon toddi* group (Haemosporida: Leucocytozoidae), with remarks on the species taxonomy of Leucocytozoids. J Parasitol 96(1):170–177

Valkiūnas G, Ashford RW, Bensch S, Killick-Kendrick R, Perkins S (2011) A cautionary note concerning *Plasmodium* in apes. Trends Parasitol 27(6):231–232

Valkiūnas G, Iezhova TA, Evans E, Carlson JS, Martinez-Gomez JE, Sehgal R (2013) Two new *Haemoproteus* (Haemosporida: Haemoproteidae) species from columbiform birds. J Parasitol 99(3):513–521

Waldenström J, Bensch S, Hasselquist D, Östman Ö (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J Parasitol 90(1):191–194. doi:10.1645/GE-3221RN

Young BE, Garvin MC, McDonald DB (1993) Blood parasites in birds from Monteverde, Costa Rica. J Wildl Dis 29(4):555–560