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Haemoproteus syrnii and other haemosporidians infecting owls from North America

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Abstract

Background Haemosporidians (Haemosporida, Apicomplexa), which include malaria parasites, are found in nearly all terrestrial ecosystems. Avian haemosporidians have been extensively studied; however, there is limited information on parasites in owls (order Strigiformes). Here, haemosporidians infecting North American owls were characterized using an integrative methodology.

Methods Taking advantage of injured/dead owls from rehabilitation centres in the central and northeastern USA, 53 individuals of *Bubo virginianus*, *Strix varia*, *Megascops asio*, *Bubo scandiacus*, *Aegolius acadicus*, and *Tyto furcata* were screened using polymerase chain reaction for all samples and microscopy for those with available blood smears. Parasite mitochondrial genomes were obtained using a long-read sequencing method (PacBio HiFi), which efficiently detects multiple infections in a single host. The relationships between parasite lineages were estimated using phylogenetic and haplotype network methods.

Results In total, 21 individuals from three species were positive by PCR: *B. virginianus* (14/17, 82.4%), *M. asio* (3/5, 60%), and *S. varia* (4/8, 50%). Two *Plasmodium*, three *Haemoproteus*, and four *Leucocytozoon* lineages were identified infecting these hosts, with one *Haemoproteus* and one *Leucocytozoon* being new to science. All positive individuals were infected with *Haemoproteus* parasites, and two *B. virginianus* had a mixed infection with *Leucocytozoon* and *Haemoproteus* species. The hSTVAR01 cytochrome b (*cytb*) lineage common in North America is linked for the first time to *Haemoproteus syrnii*. *Haemoproteus syrnii* was found in all 14 positive *B. virginianus* and two *S. varia*. Notably, all the *cytb* lineages from previously identified *H. syrnii*, based on erythrocytic stages, were not monophyletic, indicating the existence of an undescribed species. The pPADOM11 *cytb* lineage was recognized as an allele of *Plasmodium elongatum*.

Conclusion Long reads enabled the detection of mixed/co-infections. The link between genetic data and morphospecies was established in two cases. Several *Leucocytozoon* clades were observed; however, only one morphospecies, *Leucocytozoo danilewskyi*, has been described in owls. Thus, there is a need for a detailed analysis of blood stages to determine whether different owl *Haemoproteus* and *Leucocytozoon* parasites exhibit morphological differences or represent cryptic species. Overall, this study underscores the importance of high-quality molecular data in characterizing the biodiversity of haemosporidian parasites.

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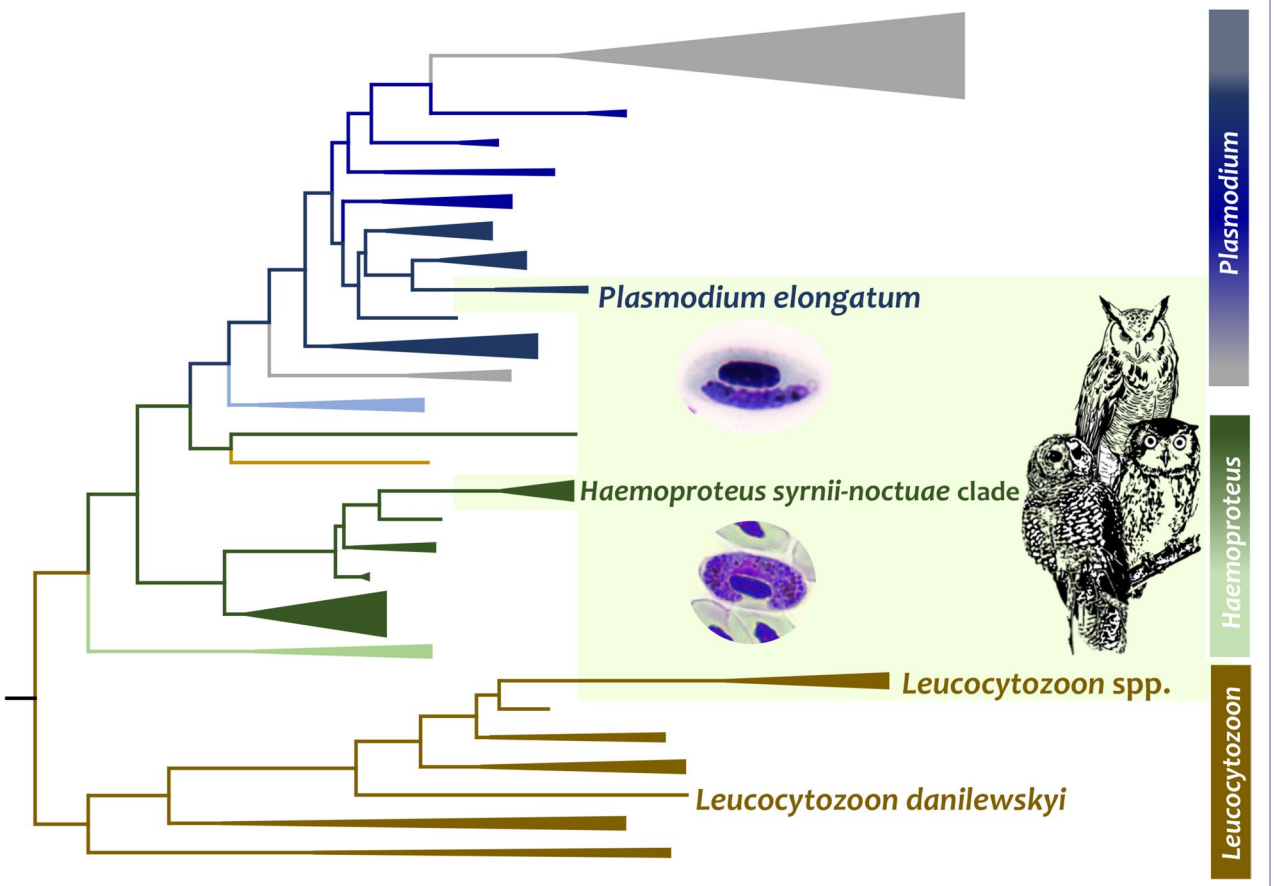
Keywords PacBio HiFi sequencing, Mitochondrial genome, Haplotype networks, hSTVAR01, *Leucocytozoon*, pPADOM11

Graphical Abstract



Background

Haemosporidian parasites (Order Haemosporida, Phylum Apicomplexa) are found in a broad range of vertebrate hosts. Their presence is often studied as a proxy for 'disease', as some species cause malaria in humans and birds [1, 2]. However, studying them solely as disease agents overlooks that birds of almost all known orders coexist in terrestrial ecosystems with these symbionts, including those species that are parasites, making them a common yet often neglected part of biodiversity [1].

Avian haemosporidians have been one of the most extensively researched groups in terms of the number of described species. They belong mainly to three families: Plasmodiidae (genus *Plasmodium*); Haemoproteidae (genus *Haemoproteus*); and Leucocytozoidae (genus *Leucocytozoon*). Among their biological differences, haemosporidians are transmitted by species of distinct vector families, all of which belong to the order Diptera [1, 3]. Most studies focus on birds that can be easily captured using mist nets, generating bias toward haemosporidians infecting passerines [4]. In recent years, however, some studies have shifted their focus to non-passerines (e.g., [4–12]) including samples from commonly hunted or live-trapped game birds [13]. Still, several avian orders have unexplored parasites; among them are all raptor species belonging to the Accipitriformes, Strigiformes, and Falconiformes orders [5–9, 11, 12, 14, 15].

Studies on raptors have been limited due to several factors, including their protected status by law, their challenging nature in terms of capture and handling, and often low population densities [8]. Here,

haemosporidians in owls (Order Strigiformes) were studied by taking advantage of opportunistic samples collected from injured/dead individuals at rehabilitation centres, and or collected by the USDA Wildlife Services from the central and northeastern regions of the USA.

Although there is a consensus on using an integrative approach in the taxonomy of avian Haemosporida, biodiversity studies have widely used molecular tools, particularly single-gene approaches targeting the mitochondrial cytochrome b gene (*cytb*). However, most studies report a small fragment of the gene [16], which is insufficient to estimate well-supported phylogenetic trees [17]. Alternatively, other studies have focused on amplifying and/or cloning almost complete parasite mitochondrial genomes (mtDNA) [11, 17–22]. The mtDNA data yield reproducible evolutionary genetic inferences, and they have been used to study haemosporidian biodiversity, biogeography, phylogenetics, and demographic processes, including population structure [11, 18–21]. Recently, an integrated long-read mtDNA protocol combining PacBio HiFi sequencing with the HmtG-PacBio Pipeline—a haplotype-aware, machine learning–driven workflow—was implemented [22], enabling the detection of parasites in hosts having either mixed infections (two genetic lineages of the same parasite species or genus) or co-infections (two distinct parasite genera), which are common in wildlife [1, 13, 17], thereby providing a robust assessment of the species pool.

Here, this integrated long-read mtDNA protocol was applied to characterize assemblages of owl haemosporidian species. Diverse haemosporidian lineages were found in owls, and two new lineages (one *Haemoproteus* and one *Leucocytozoon*) are reported. By combining microscopy and molecular data, a previously reported hSTVAR01 lineage was assigned to the morphospecies *Haemoproteus* (*Parahaemoproteus*) *syrnii*, a common owl parasite first described in Europe. This lineage is widely distributed in North America and was found here in *Bubo virginianus* and *Strix varia* as part of co-infections with lineages of *Leucocytozoon* spp. The mitochondrial genomes of *H. syrnii* are reported here for the first time, together with an extensive revision of the molecular data available for owls around the world in the MalAvi database [16]. Molecular analyses revealed that several *cytb* lineages previously considered to be *H. syrnii* were not part of a monophyletic group. Overall, this study contributes new knowledge to a better understanding of haemosporidian parasite biodiversity infecting owls.

Methods

Samples, DNA extraction, and haemosporidian screening

Fifty-three samples were collected from 2019 to 2021 from owls presented to wildlife rehabilitation centres (N = 29) and Project SNOWstorm (N = 22), or captured dead by the USDA Wildlife Services (N = 2), as part of an ongoing collaboration to study haemosporidian parasites circulating in raptor populations from North America (Delaware, Illinois, Maryland, New Jersey, Maine, Massachusetts, Minnesota, and Pennsylvania states). Samples were collected during routine, diagnostic necropsies (of birds found dead, died in rehabilitation, or euthanized in rehabilitation due to presenting condition) or during blood collection as part of routine physical examinations of live rehabilitation or ambassador birds. All methods were performed in accordance with relevant guidelines and regulations.

The species included in this study were: Great Horned Owl *B. virginianus*, Barred Owl *S. varia*, Eastern Screech Owl *Megascops asio*, Snowy Owl *Bubo scandiacus*, Northern Saw-whet Owl *Aegolius acadicus*, and Barn Owl *Tyto furcata* (Table 1). Detailed information about the samples is provided in Additional file 1: Table S1A. Only blood or blood and/or liver samples were collected, depending on the animal's condition. Blood samples were preserved on protein saver cards (Whatman 903, Whatman, Cardiff, UK) for molecular analysis. For some individuals (N = 5), two to three thin smears were prepared for microscopy analysis immediately after they were received at the rehabilitation centres to ensure that the observed infections were not acquired in captivity. Smears were immediately dried, then fixed with 100% methanol for 1 min and stained with 10% Giemsa (pH 7.2) for 60 min. Unfortunately, thin smears could not be prepared for all the samples, given that many of them were found already dead (see Additional file 1: Table S1A). Liver tissues were collected from dead animals and preserved in 95% ethanol.

Genomic DNA was extracted from whole blood or liver using the DNeasy Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. Then, the extracted DNA was screened for haemosporidian parasites using a nested polymerase chain reaction (nested-PCR), with oligos targeting the complete *cytb* gene, which have been used in previous studies [17, 21, 23–25]. Briefly, primary PCR amplifications were carried out in a 50 µL volume with 2 µL of total genomic DNA, 2.5 mM MgCl2, 1 × PCR buffer, 0.25 mM of each deoxynucleoside triphosphate, 0.4 µM of each primer, and 0.03 U/µL AmpliTaq polymerase (Applied Biosystems, Thermo Fisher Scientific, USA). External PCR oligos forward AE298 (5′-TGT AAT GCC TAG ACG TAT TCC 3′) and reverse AE299 (5′-GT CAA WCA AAC ATG AAT ATA GAC 3′), and internal oligos forward AE064 (5′-T CTA TTA ATT TAG YWA AAG CAC 3′) and reverse AE066 (5′-G CTT GGG AGC TGT AAT

Table 1 Haemosporidians detected by PCR and microscopy in owls (Strigiformes: Strigidae and Tytonidae families) from United States

Owl Species	N	Total Positive (%)	*Plasmodium* (Lineage, N)	*Haemoproteus* (Lineage, N)	*Leucocytozoon* (Lineage, N)	Origin (N)
Great Horned Owl (*Bubo virginianus*)	17	14 (82.4)		*H. syrnii* (STVAR01*, 14)	*L.* sp. (BUVIR02, 1) *L.* sp. (BUVIR06, 1), *L.* sp. (**BUVIR10**, 1), *L.* sp. (STOCC16, 1)	DE (2)/IL(2)/ MD(4)/ NJ (5)/PA (5)
Eastern Screech Owl (*Megascops asio*)	5	3 (60.0)	*P.* sp. (WW3, 1) *P. elongatum* (PADOM11)*	*H.* sp. (**MEGASI01**, 2)		NJ (3)/PA (2)
Barred Owl (*Strix varia*)	8	4 (50.0)		*H. syrnii* (STVAR01, 2) *H.* sp. (STVAR06, 2)		DE (3)/MD (2)/ NJ (2)/PA (2)
Snowy owl (*Bubo scandiacus*)	21	0 (0)				MA (12)/ME (7)/ NJ (1)/PA (1)
Northern Saw-whet Owl (*Aegolius acadicus*)	1	0 (0)				MN (1)
Barn owl (*Tyto furcata*)	1	0 (0)				PA (1)
Total	**53**	**21 (39.6)**				

*Parasites detected by microscopy and PCR

DE Delaware, *IL* Illinois, *MD* Maryland, *ME* Maine, *MN* Minnesota, *NJ* New Jersey, *PA* Pennsylvania

New lineages are shown in bold

CAT AAT 3′) were used. Primary PCR conditions were a partial denaturation at 94 °C for 4 min and 36 cycles with 1 min at 94 °C, 1 min at 53 °C, and 2 min extension step at 72 °C. In the last cycle, a final extension step of 10 min at 72 °C was added. The nested PCR mix and conditions were the same as those for the primary one but using 1 µL of the primary PCR product and an annealing temperature of 56 °C. Amplified products (50 µL) were excised from agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). Both strands of the *cytb* gene were directly sequenced at Genewiz (Azenta Life Sciences, New Jersey, USA). All *cytb* gene sequences obtained here were deposited in GenBank under the accession numbers PV948495-PV948515.

Microscopic examination was carried out on blood smears that were deemed of high quality [3] using a Leica DM750 light microscope (Leica Microsystems, USA) equipped with a Leica ICC50 W camera and Leica Acquire software to capture images. On those, 200 microscopic fields at low magnification (×400), and 200 fields at high magnification (×1000) were examined. Intensity of infection was estimated as the number of parasites/20,000 erythrocytes counted at high magnification [26]. Measurements were made using ImageJ [27] and, for the *H. syrnii* detected here, they were compared to values provided by Valkiūnas [3] for *H. syrnii* infecting a Ural Owl *Strix uralensis* using Student's *t*-tests.

Haemosporidian mitochondrial genome PacBio HiFi protocol

Haemosporidian mitochondrial genomes were amplified using TaKaRa LA Taq Polymerase (TaKaRa Mirus Bio Inc.) according to the manufacturer's directions [18] and sequenced using a PacBio HiFi protocol [22]. In brief, three independent PCRs were performed for each positive sample using 2 µL of DNA and a unique oligo combination for each sample (see Table 2 in [22]). All PCR reactions were carried out in 50 µL volumes, and negative (dH_2O) and positive controls (*Plasmodium vivax*) were included. Amplification conditions for all PCRs were a partial denaturation at 94 °C for 1 min and 30 cycles with 30 s at 94 °C and 7 min at 68 °C, followed by a final extension of 10 min at 72 °C. PCR products were visualized in 1% LE analytical grade agarose (Promega Corporation, USA) gels stained with GelRed® Nucleic Acid Gel Stain (Biotiun, San Francisco-California, USA). All three independent PCR products (50 µL) were excised from the gel (bands of ~6 kb) and purified using the QIAquick Gel extraction kit (Qiagen, GmbH, Hilden, Germany).

Subsequently, all purified PCR products (50 µL x replicate x samples) were pooled in a clean 2.0 mL DNA LoBind microcentrifuge tube (Eppendorf, Hamburg, Germany) and concentrated to 200 µL. The total DNA concentration was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Massachusetts, USA), with a total amount of DNA in the pool of 5720 ng (28.6 ng/µL). Then, the pool was dried and sent to the DNA Services

Table 2 Morphometric parameters of gametocytes of *Haemoproteus syrnii* and red blood cells of Great Horned Owl (*Bubo virginianus*), (this study) and values provided by [3] for *H. syrnii* infections in Ural Owl *Strix uralensis*

Feature	Measurements	
	This study	Valkiūnas, 2005 [3]
Uninfected erythrocyte	n = 20	N = 32
Length	13.7 ± 0.7 (12.3–14.9)	13.4 ± 0.7 (11.4–15.0)
Width	7.35 ± 0.5 (6.4–8.3)	7.4 ± 0.2 (6.3–8.6)
Area	76.7 ± 8.0 (61.4–92.6)	NM
Uninfected erythrocyte nucleus	n = 20	n = 31
Length[a]	6.5 ± 0.4 (5.9–7.4)	5.3 ± 0.1 (4.0–7.7)
Width[a]	2.6 ± 0.3 (2.0–3.2)	2.5 ± 0.1 (2.0–3.5)
Area	12.8 ± 1.1 (10.9–15.4)	NM
Macrogametocyte	n = 10	n = 24
Infected erythrocyte		
Length[a]	14.5 ± 0.8 (13.2–16.0)	14.0 ± 0.9 (11.9–16.0)
Width	7.1 ± 0.6 (6.0–8.0)	7.3 ± 0.4 (6.4–8.8)
Area	86.6 ± 7.8 (72.3–100.2)	NM
Infected erythrocyte nucleus		
Length[a]	5.6 ± 0.6 (4.9–6.9)	4.4 ± 0.2 (3.0–6.1)
Width	2.3 ± 0.3 (2.0–3.2)	2.2 ± 0.1 (2.0–3.4)
Area	10.5 ± 1.0 (9.4–12.5)	NM
Gametocyte		
Length[a]	17.5 ± 2.7 (13.7–21.9)	15.0 ± 1.1 (12.4–18.8)
Width[a]	2.5 ± 0.5 (1.6–3.3)	3.8 ± 0.4 (2.7–4.8)
Area	49.8 ± 9.8 (39.6–75.7)	NM
Gametocyte nucleus		
Length	3.4 ± 0.4 (2.5–4.1)	3.3 ± 0.3 (2.1–4.1)
Width	1.9 ± 0.3 (1.3–2.3)	2.0 ± 0.2 (1.0–3.0)
Pigment granules[a]	30.7 ± 4.9 (22–41)	18.6 ± 3.3 (13–26)
NDR[a]	0.7 ± 0.2 (0.3–1.0)	0.5 ± 0.1 (0.2–0.7)
Microgametocyte	n = 3	n = 17
Infected erythrocyte		
Length[a]	15.4 ± 0.5 (14.8–15.8)	14.2 ± 0.8 (11.9–16.4)
Width[a]	7.5 ± 0.3 (7.0–7.7)	6.9 ± 0.5 (6.4–9.0)
Area	93.2 ± 3.5 (88.2–96.0)	NM
Infected erythrocyte nucleus		
Length[a]	6.1 ± 0.1 (5.9–6.2)	5.0 ± 0.2 (4.0–6.4)
Width	2.3 ± 0.2 (2.2–2.6)	2.0 ± 0.1 (1.4–2.6)
Area	11.1 ± 0.8 (10.6–12.2)	NM
Gametocyte		
Length	16.4 ± 2.5 (13.4–19.5)	13.8 ± 1.0 (12.0–16.9)
Width[a]	2.3 ± 0.1 (2.1–2.5)	3.7 ± 0.5 (2.7–4.6)
Area	42.9 ± 5.0 (38.4–50.0)	NM
Gametocyte nucleus		
Length[a]	10.0 ± 0.5 (9.4–10.6)	8.0 ± 0.4 (5.5–11.1)
Width[a]	2.1 ± 0.1 (2.0–2.2)	3.1 ± 0.2 (1.6–4.2)
Pigment granules	16.0 ± 5 (11–21)	15.1 ± 2.9 (11–22)
NDR[a]	0.8 ± 0.1 (0.8–0.9)	0.4 ± 0.1 (0.2–0.6)

Table 2 (continued)

All measurements are given in micrometres except for nucleus displacement ration (NDR). Mean ± standard deviation, followed by minimum and maximum values, are provided. NDR was calculated according to [50]. NM = not measured

[a] Statistically significant differences between measurements reported here and those provided by [3]

of the University of Illinois at Urbana-Champaign, Roy J. Carver Biotechnology Center (Urbana, IL 61801) for the AMPure PB bead purification, multiplexed SMRTbell® library preparation, sequencing, and demultiplexing to pull reads from the positive owl samples. Amplicons were converted to a library with the SMRTBell Express Template Prep kit 3.0. Then, it was sequenced on one SMRTcell 8M on a PacBio Sequel IIe using the CCS sequencing mode and a 30hs movie time. CCS analysis was done using SMRTLink V11.0 with the following parameters: ccs-min-passes 3-min-rq 0.999 and lima-ccs-preset HIFI-ASYMMETRIC-split-bam-named. Then, a haemosporidian mitochondrial genome PacBio HiFi pipeline (HmtG-PacBio Pipeline) that utilizes a machine learning method [22] was employed to analyze the data obtained from PacBio HiFi sequencing. An updated version pipeline is available on GitHub at https://github.com/EscalanteLab/HmtG-PacBio-Pipeline.git (see Fig. 1 in [22]). Before the execution of the HmtG-PacBio pipeline, a preliminary step involving the filtering and cleaning of reads was implemented in a new version. This preprocessing facilitates the detection and discarding of reads with sequencing errors by masking low-quality sites (Qscore < 30) and removing reads with more than 6 sites with sequencing errors (PacBio error rate 0.1%).

In addition, the haemosporidian mitochondrial genome for each unique haemosporidian species found in the owl samples was also amplified using the same PCR protocol and cloned [18] to compare/validate the results with the sequences obtained with the HmtG-PacBio HiFi protocol [22]. The mtDNA genomes for *Haemoproteus tinnunculus* (hFALSUB01), *Haemoproteus homopicae* (hGAGLA07), and *Plasmodium matutinum* (pLINN1) were also obtained by this protocol to increase the number of the mt genomes linked to well-known parasites described to the morphospecies level. Briefly, at least two independent PCR products for all these samples were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), and four clones were sequenced from each individual. Both strands were sequenced for each clone at Genewiz (Azenta Life Sciences, New Jersey, USA). All mt genome sequences generated in this study were deposited in GenBank under the accession numbers PV948475-PV948494 and added to the set of mitochondrial genome sequences available on the GitHub at https://github.com/EscalanteLab/HmtG-PacBio-Pipel

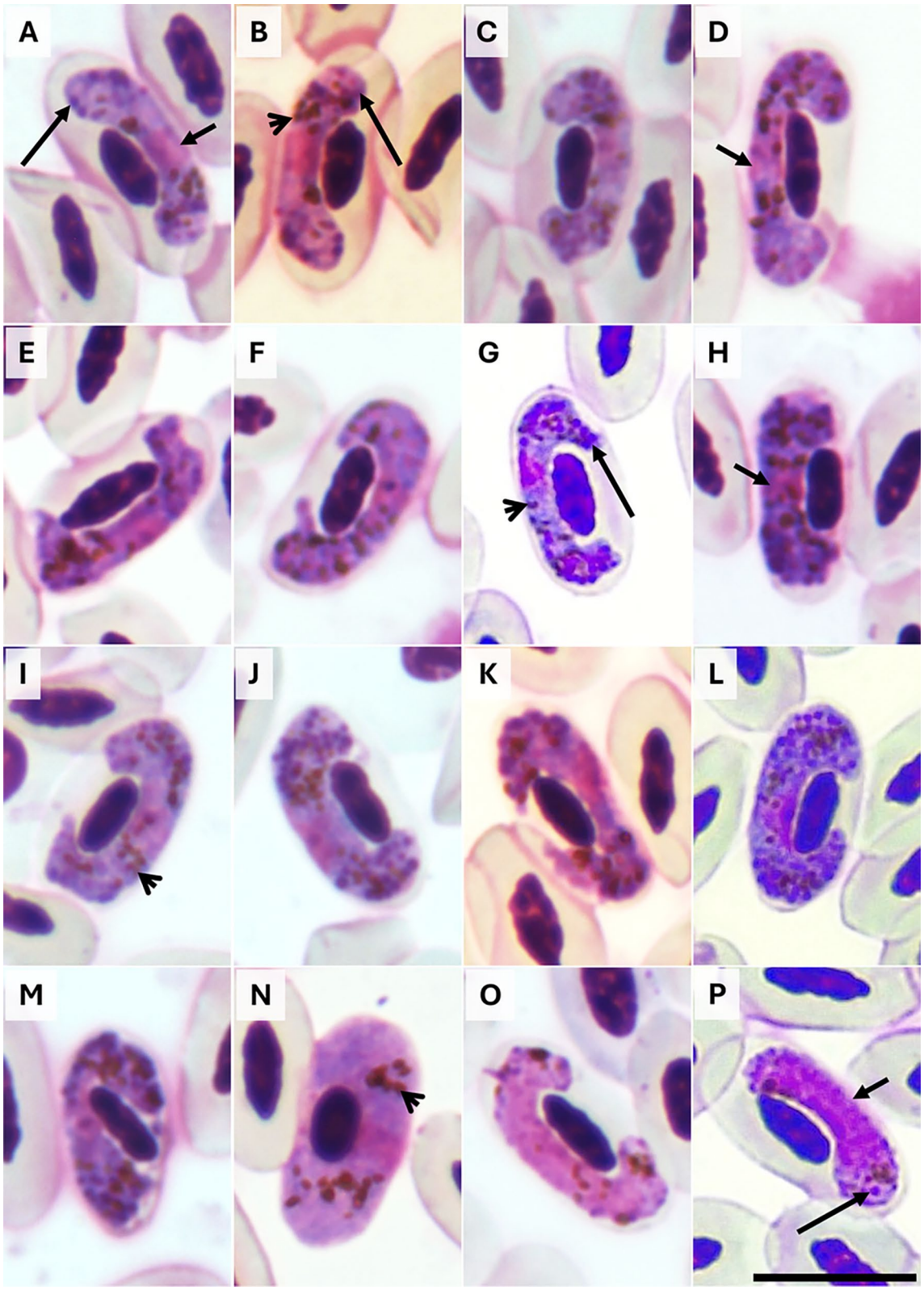


Fig. 1 *Haemoproteus syrnii* hSTVAR01 from a Great Horned Owl (*Bubo virginianus*) sampled in Mercer County, NJ in 2019. **A**–**J**, growing macrogametocytes; **K**–**N**, mature macrogametocytes; **O**–**P**, growing microgametocytes. Short arrows, parasite nuclei; long arrows, volutin granules; arrowhead, haemozoin granules. Giemsa-stained thin blood film. Scale bar = 10 µm

ine.git, which compilate most of the published mt genome sequences already deposited in GenBank [28].

To compare the owl parasite mtDNA genomes obtained here with some known lineages, the mtDNA genomes of four *Haemoproteus* spp. and two *Leucocytozoon* spp. lineages from individuals collected in Europe were obtained using a nested PCR approach developed recently by Harl et al. [29]. The four *Haemoproteus* lineages were *H. syrnii* hSTAL2 (AH1912) and hCULKIB01 (AH0233) from the Ural Owl *S. uralensis*, hASIOTU04 (AH0144) from the Long-eared Owl *Asio otus*, and *Haemoproteus noctuae* hCIRCUM01 (AH0031) from *A. otus*. The two *Leucocytozoon* lineages were lATNO1 (AH0392) from the Little Owl *Athene noctua* and lSTAL10 (AH0172) from a *S. uralensis*. The samples originally were part of a batch of owls from Austria screened for haemosporidian parasites as described in Ilgūnas et al. [9]. The mt genomes were amplified by long-range PCR using lineage-specific primers placed within the *cytb* but pointing outwards, followed by five nested PCRs using the long-range PCR primers and primers placed in conserved genome regions producing overlapping fragments. The primers for the long-range PCRs were mt_F2_STAL2 (5′-GGA TTG GAT GTC AAT TAC CTC AAT C-3′) and mt_R5_STAL2 (5′-ACA AAT GTA GCA CCA GTA GCA T-3′) for the four *Haemoproteus* genomes, mt_F2_ATNO1_F2 (5′-ACT AAT CCT TTA GGG TAT GAT ACT C-3′) and mt_R5_ATNO1 (5′-TCC AGG ATA ATG GTA AAT AGG AG-3′) for *Leucocytozoon* sp. lATNO1, and mt_F2_STAL10_F2 (5′-ATT TTC CCA TTT ATA GCA CTA GTT GT-3′) and mt_R5_STAL10 (5′-TAC AAA AGA TGC ACC GGT TG-3′) for *Leucocytozoon* sp. lATNO1. The long-range and nested PCRs were performed as described in Harl et al. [29]. The mt genomes obtained using this method were deposited in GenBank under the accession numbers PV839541 to PV839546.

Phylogenetic analyses and haplotype networks

After validating the quality of the sequences obtained by the two methods (HmtG-PacBio HiFi sequencing and Sanger sequencing/cloning), and comparing them against the sequences available in GenBank [28] and MalAvi [16] databases using BLAST, three phylogenetic analyses were performed using all sequences obtained in this study (*cytb* gene N = 21, mtDNA N = 17; Table 1) as well as sequences from well-known lineages or parasite species based on morphology [3, 18, 30] that were available in GenBank [28] and MalAvi [16] databases at the time of this study (July 2025). For these analyses, the alignments were constructed using MAFFT v.7 [31] with manual editing. First, an alignment was done using the parasite mtDNA genome sequences (5134 bp excluding gaps) available for 149 haemosporidians belonging to five genera (*Leucocytozoon*, *Haemocystidium*, *Haemoproteus*, *Hepatocystis*, and *Plasmodium*). Second, a *cytb* gene alignment (N = 77, 436 bp excluding gaps) was performed using the small commonly used *cytb* gene fragment from *Haemoproteus* (*Parahaemoproteus*) species [30]. Sequences from the genera *Haemoproteus* (*Haemoproteus*) and *Leucocytozoon (Akiba) caulleryi* were used as an outgroup for this alignment. A third alignment was generated (N = 87, 407 bp excluding gaps) using the same *cytb* fragment from *Leucocytozoon* species/lineages [3, 32], and sequences from *Haemoproteus* (*Haemoproteus*) spp., *Haemocystidium* spp., *Haemoproteus catharti*, *Haemoproteus pulcher*, and *Leucocytozoon* (*Akiba*) *caulleryi* were included as an outgroup.

Then, phylogenetic relationships were inferred on these three alignments using a maximum likelihood (ML) method implemented in IQ-TREE v2.3.1 [33] and a Bayesian method implemented in MrBayes v3.2.7 with the default priors [34]. For both analyses, a general time-reversible model with gamma-distributed substitution rates and a proportion of invariant sites (GTR + Γ + I) was used, as the best substitution model determined by ModelFinder [35] as implemented in IQ-TREE [33]. In the case of ML, support values were generated through Ultrafast bootstrap approximation (UFBoot) using 1000 replicates [36]. In the case of the Bayesian method, posterior probabilities for the nodes were estimated from two independent chains of 4×10^6 Markov Chain Monte Carlo (MCMC) steps by sampling every 500 generations. Convergence was assumed when the value of the potential scale reduction factor (PSRF) was between 1.00 and 1.02, and the average standard deviation of the posterior probability was < 0.01 [34]. A "burn-in" of 25% of the sample was discarded. Phylogenetic trees were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Parasite species names, GenBank accession numbers, and lineage name of all sequences used in the analyses are shown in the phylogenetic trees. Average evolutionary distance over sequence pairs of *Haemoproteus* and *Leucocytozoon* spp. found in owls were estimated using the Tamura–Nei substitution model implemented in MEGA v7.0.14 [37].

To study the genetic relationships among worldwide owl *cytb* gene lineages via haplotype network analysis, three more alignments were performed with MAFFT v.7 [31] using all *Haemoproteus* and *Leucocytozoon* sequences available for owls in the MalAvi database (Additional file 1: Table S1B). Parasite species included in each alignment were: (1) 209 sequences for *Haemoproteus* spp. lineages and related morphospecies from owls (464 bp excluding gaps); (2) 113 sequences that include all *Haemoproteus* spp. lineages that shared a recent common ancestor to *H. syrnii* hSTAL2 lineage, the most

common species found in this study as determined from the haplotype network analysis carried out with the first alignment (464 bp excluding gaps); and (3) 143 *Leucocytozoon* spp. lineages found in owls (400 bp excluding gaps). Then, these alignments were used to estimate median-joining networks using PopArt 1.7 based on single-nucleotide variation (SNV) [38]. Transversions were set equal to transitions, and the epsilon parameter was set equal to 0 with only one round of star contraction, which collapses star-like structures in the network into single nodes.

Results

Molecular and microscopic detection of haemosporidian parasites

Twenty-one out of 53 (39.6%) owl samples collected were found positive for haemosporidian parasites (Table 1) using a nested-PCR protocol [17]. Only three of the six owl species (*B. virginianus*, *S. varia*, and *M. asio*) had haemosporidian parasites. The result of BLASTn using the partial *cytb* gene and mt genome sequences showed infections of two *Plasmodium* lineages (pWW3/pPADOM11), two *Haemoproteus* lineages (hSTVAR01/hSTVAR06), and three *Leucocytozoon* lineages (lBUVIR02/lBUVIR06/lSTOCC16) (Table 1) plus two new lineages (*Haemoproteus* hMEGASI01/*Leucocytozoon* lBUVIR10). Given that this is an opportunistic study, the actual prevalence of haemosporidians cannot be estimated for each owl species. However, 82.4% (14/17) of the *B. virginianus* were infected with *Haemoproteus* sp. hSTVAR01, two of which were co-infected with *Leucocytozoon* lineages (Table 1, Additional file 1: Table S1A). One individual had a co-infection with *Haemoproteus* sp. hSTVAR01 and *Leucocytozoon* sp. lBUVIR10 (a new lineage), and the other also had the hSTVAR01 lineage and three *Leucocytozoon* lineages (Table 1, Additional file 1: Table S1A, Additional file 2: Figure S1) as was detected by the HmtG-PacBio HiFi protocol.

A *B. virginianus* infected with *Haemoproteus* sp. hSTVAR01 was also positive by microscopy analysis. Although parasitaemia was low (0.05% of erythrocytes infected), a morphological characterization of this parasite as *H. syrnii* was possible (Fig. 1a–p, Table 2) based on the following similarities with descriptions provided by Valkiūnas [3] and Karadjian et al. [39]: both macrogametocytes and microgametocytes display prominent roundish volutin granules scattered throughout the cytoplasm (Fig. 1a, g, p); the gametocyte nuclei have a median or submedian position (Fig. 1a–p), their extremities encircle the host cell nucleus without completely surrounding it, and they markedly displace the host cell nuclei (Fig. 1h, n). Although few microgametocytes were found, their volutin and pigment granules were accumulated at the gametocyte extremities, corresponding to *H. syrnii* description (Fig. 1o, p). Growing macro- and microgametocytes are not appressed to the host cell nuclei (Fig. 1a, b, o, p). Therefore, the lineage hSTVAR01 belong to the *H. syrnii* group of lineages.

Three *M. asio* (60%) were infected, and the new *Haemoproteus* lineage hMEGASI01 was detected in two individuals. In addition, one *M. asio* had a mixed infection with two *Plasmodium* lineages (pWW3 and pPADOM11), and each lineage was detected by a different molecular method. The *cytb* gene protocol detected pWW3 [17], while pPADOM11 was detected by the PacBio HiFi protocol (Table 1) [22]. Also, via microscopy, two different *Plasmodium* parasites were confirmed in this individual (Fig. 2a–p). Overall parasitaemia was 0.3%, and the found blood stages corresponded to *Plasmodium elongatum* (Fig. 2a–o): mainly, trophozoites (Fig. 2a–d) and meronts (Fig. 2e–h) developed in immature erythrocytes; meronts formed less than 10 elongated merozoites; gametocytes (Fig. 2i–o) were elongated and only slightly displaced the nucleus of the host cell in approximately 60% of the cases. In the same blood smear from this host, a large roundish gametocyte of a second parasite was detected, which was characteristic of the subgenus *Plasmodium* (*Haemamoeba*) sp. (Fig. 2p). In the case of the *S. varia*, four out of 8 (50%) had haemosporidians confirmed only by nested-PCR/HmtG-PacBio HiFi protocol; two individuals were infected with hSTVAR01 and two with hSTVAR06 (Table 1).

Phylogenetic analyses and haplotype networks

The phylogenetic relationships of parasite lineages based on their mtDNA are shown in Fig. 3. This is an unrooted tree and includes the eight haemosporidian lineages reported in this study and those sequenced as reference (N = 5). *Plasmodium* spp. pPADOM11 and pWW3 were only detected in mixed infection in one individual of *M. asio* using both microscopy and PCRs. Given the parasite mtDNA data available so far, the pPADOM11 lineage was found to share a recent common ancestor with *P. elongatum* (KY653801: pGRW06, Fig. 3), with a genetic distance of 0.005 ± 0.001. In the case of pWW3, its mt genome could not be rescued with the protocols used here (very low parasitemia), and only its *cytb* gene sequence was available. However, it seems related to *Plasmodium lutzi* DIGLAF01 (KF5372760) with a genetic distance of 0.019 ± 0.006, a parasite that belongs to the subgenus *P.* (*Haemamoeba*). This finding is consistent with the morphological data obtained from the blood smear.

Regarding the *Haemoproteus* lineages, both phylogenetic hypotheses using the nearly complete mtDNA genomes (Fig. 3) and the partial *cytb* gene (Fig. 4) indicated that the hSTVAR01 lineage shares a recent common

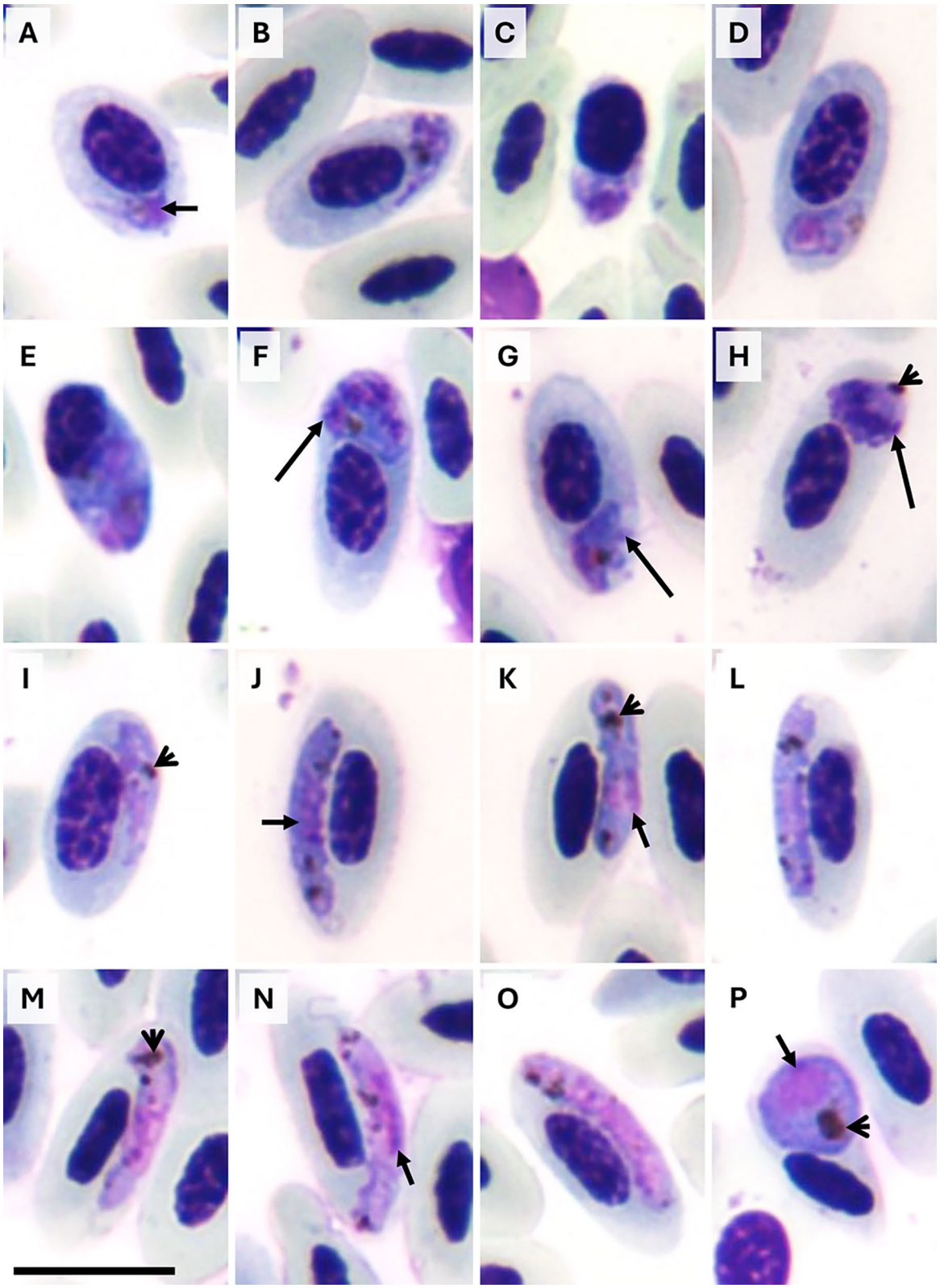


Fig. 2 *Plasmodium elongatum* pPADOM11 (Fig. A-O) and *Plasmodium* sp. pWW3 (Fig. P) infecting an Eastern Screech Owl (*Megascops asio*) sampled in Hamilton Township, Mercer Co., NJ in 2020. **A**–**D**, trophozoites; **E**–**H**, meronts; **I**–**L**, macrogametocites; **M**–**O**, **P**, microgametocytes. Short arrows, parasite nuclei; long arrows, merozoites; arrowhead, hemozoin granules. Giemsa-stained thin blood film. Scale bar = 10 µm

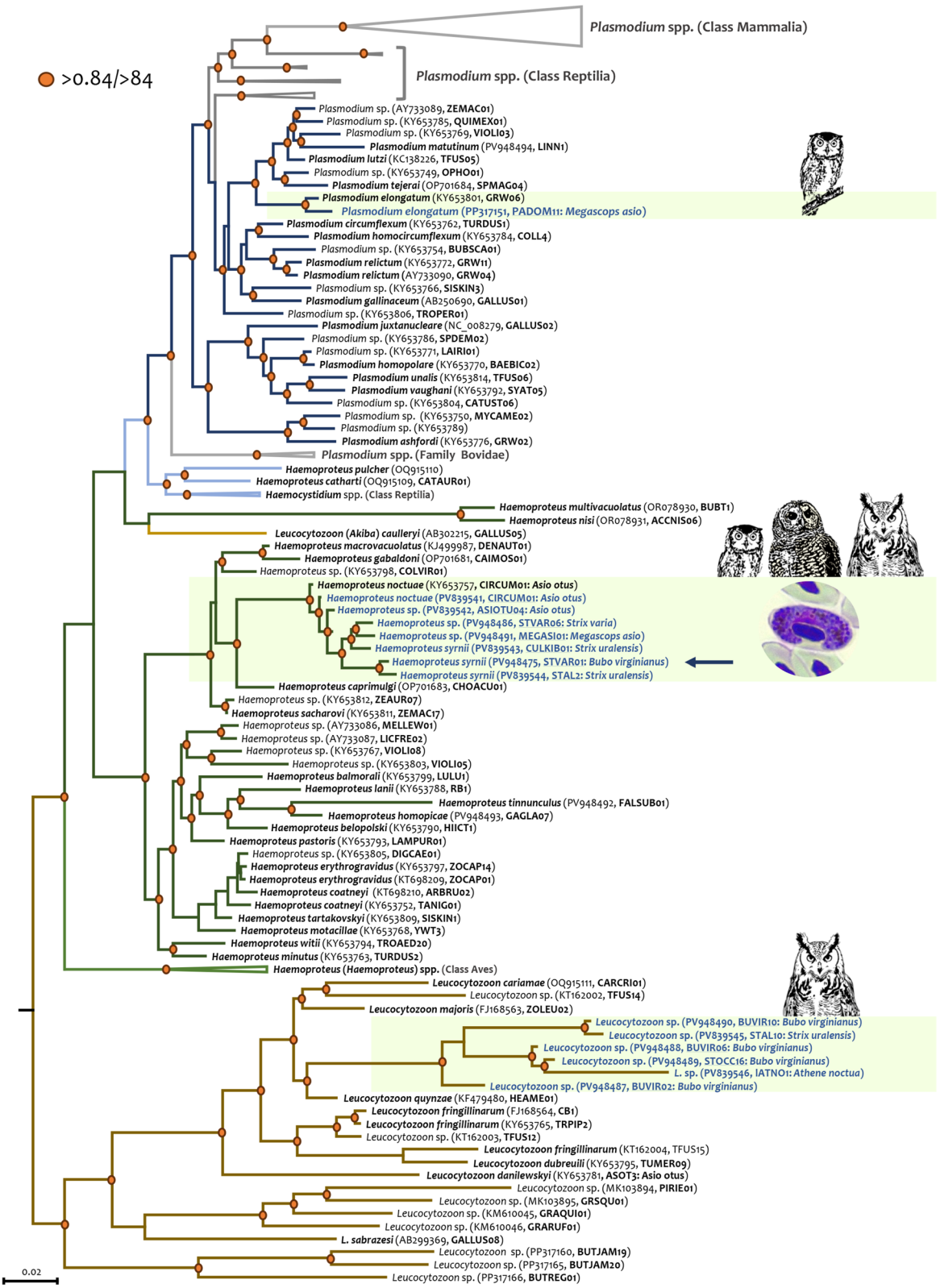


Fig. 3 Phylogenetic hypotheses of Haemosporida parasites based on mtDNA genomes (N = 149, 5134 bp excluding gaps). Branch colors indicate different genera/hosts. GenBank accession numbers and MalAvi lineage for all parasite sequences used in these analyses are provided in parentheses. Lineages/species reported in this study are written in blue. Orange dots at the nodes mean a posterior probability greater than 0.84 and a bootstrap greater than 84

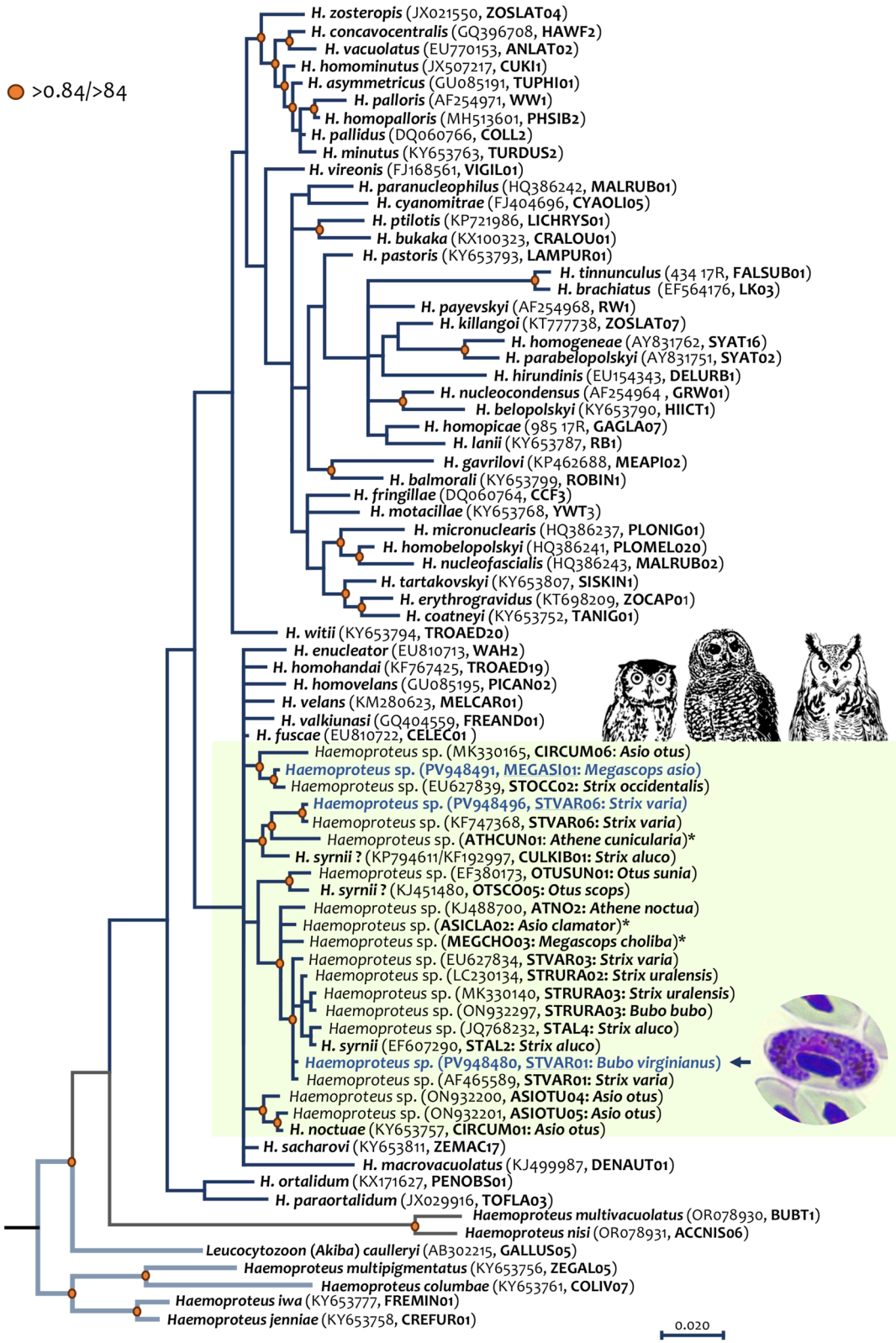


Fig. 4 Phylogenetic hypotheses of genus *Haemoproteus* based on partial *cytb* gene sequences (N = 77, 436 bp excluding gaps). Branch colors indicate different genera/hosts. GenBank accession numbers and MalAvi lineage for all parasite sequences used are provided in parentheses. Lineages/species reported in this study are written in blue. Orange dots at the nodes mean a posterior probability greater than 0.84 and a bootstrap greater than 84. Sequences from Brazilian owls are shown with an "*"

ancestor with *H. syrnii* hSTAL2 (EF607290) reported in Tawny Owl *Strix aluco* from Europe (Additional file 1: Table S1B, [39]). The estimated genetic distances using the partial *cytb* gene and mt genome between hSTAL2 (the reference lineage for *H. syrnii*) and hSTVAR01 were very low (0.004 ± 0.003 and 0.006 ± 0.001, respectively; Additional file 1: Table S1C), with only two synonymous substitutions found between them. However, the two other lineages considered as *H. syrnii* based on morphology with molecular data available (hOTSCO05, KJ451480: *Otus scops* [40] and hCULKIB01, KP794611: *S. aluco* [41]) do not share a common ancestor with the hSTAL2 and hSTVAR01 lineages (Figs. 4 and 5). The genetic distances between these lineages (hOTSCO05/hCULKIB01 *vs.* hSTAL2/hSTVAR01) were almost 9–10 times greater than those between hSTAL2 and hSTVAR01 (Additional file 1: Table S1C). The relationship between these lineages is also depicted in the haplotype networks estimated with all the *Haemoproteus* spp. reported in owls (Figs. 5 and 6). The haplotype networks also included the species that formed a clade in the *cytb* phylogenetic tree (Figs. 4 and 5) or the *Haemoproteus* spp. considered closely related to *H. syrnii* (including sequences from lineages found in owls from Brazil [42], Fig. 6). The lineages hSTAL4, hSTRURA02, hSTRURA03, hSTVAR01, and hSTVAR03 were the closest to the lineage originally described as *H. syrnii* hSTAL2 [39] (Figs. 4, 5 and 6, Additional file 1: Table S1C).

Given the results from the estimated genetic distances and haplotype networks, any of the lineages reported in Brazil as *H. syrnii* (e.g., hATHCUN01, hMEGCHO03, and hASICLA02; [42]) do not share a recent common ancestor with the original *H. syrnii* hSTAL2 (Figs. 4, 5 and 6; Additional file 1: Table S1C). The closest ancestors to hATHCUN01 (hypothetical *H. syrnii*), found in the Burrowing Owl *Athene cunicularia* from Brazil, were *Haemoproteus* sp. hSTVAR06 found here in two *S. varia*, the proposed *H. syrnii* lineage hCULKIB01 detected in a *S. aluco* from Europe (Fig. 4), and the hPLAMIN01 lineage found in two *S. uralensis* from Asia (Fig. 5).

On the other hand, the new *Haemoproteus* sp. hMEGASI01 lineage found in *M. asio* was closely related to *Haemoproteus* sp. hSTOCC02 (genetic distance = 0.002 ± 0.002) reported before in Spotted Owl

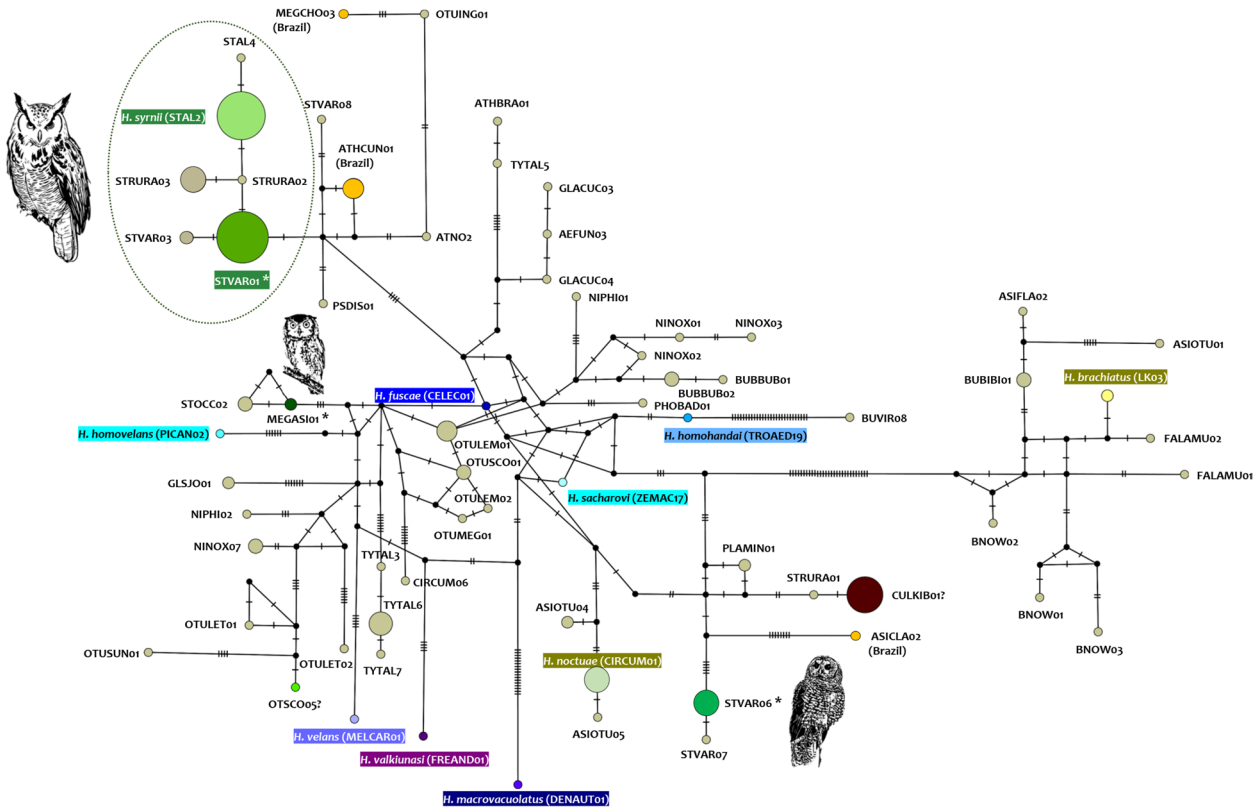


Fig. 5 Minimum spanning network for *Haemoproteus* parasites based on partial *cytb* gene lineages (N = 209, 464 bp excluding gaps) identified from owls and closely related morphospecies. Circles are drawn proportionally to the frequency at which lineages were reported in MalAvi database. Lines between circles have the number of mutations that separate two lineages. Lineages of *Haemoproteus syrnii* group are indicated in green, and other morphospecies are indicated in different colors. Lineages reported in this study are shown with an "*"

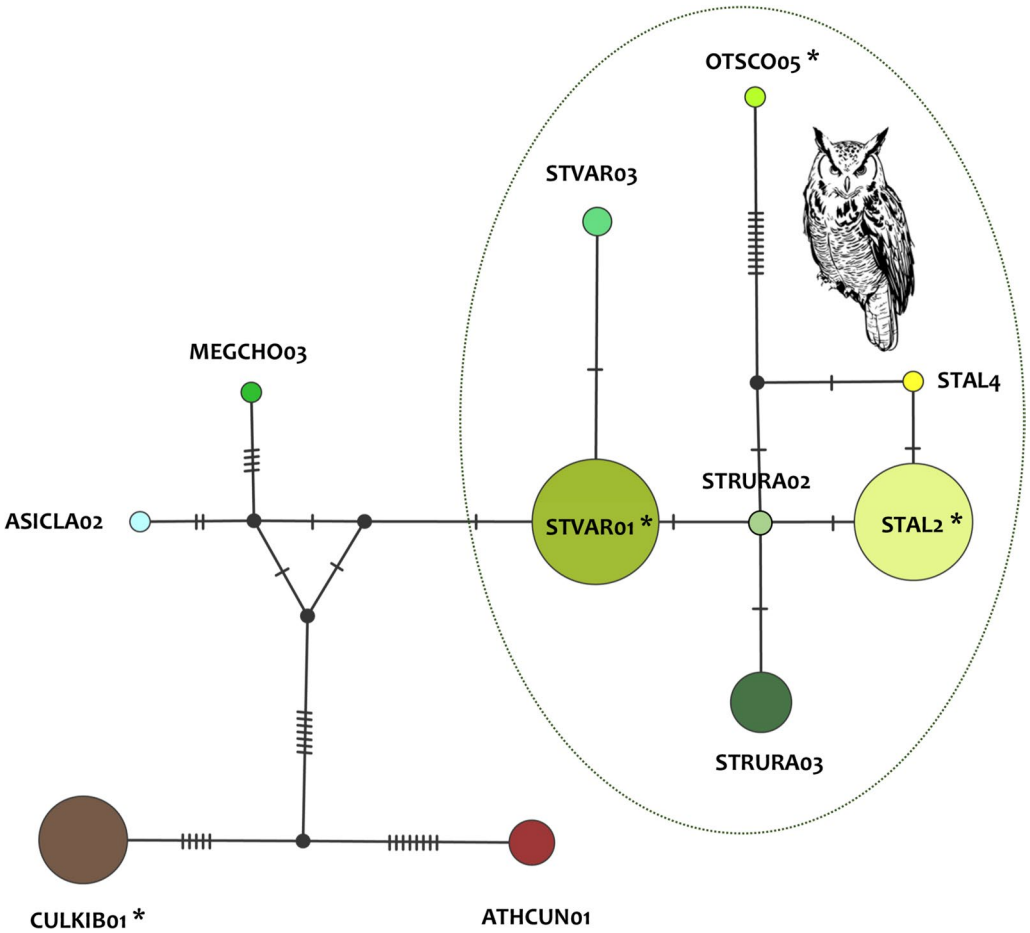


Fig. 6 Minimum spanning network for *Haemoproteus syrnii* group based on partial *cytb* gene lineages (N = 113, 464 bp excluding gaps) identified from owls. The dataset includes all *Haemoproteus* spp. lineages closely related to *H. syrnii* hSTAL2, the most common lineage found in this study. Circles are drawn proportional to the frequency at which lineages were detected. Lines between circles have the number of mutations that separate two lineages. Lineages *of H. syrnii* group are indicated in green. Lineages reported as *H. syrnii* in the literature are shown with an "*"

Strix occidentalis and *S. varia* from North America [43] (Figs. 4 and 5; Additional file 1: Table S1B). All *Haemoproteus* spp. found in owls with available mtDNA genomes formed a monophyletic group that shares a common ancestor with *Haemoproteus caprimulgi* (OP701683, hCHOACU01) and other *Haemoproteus* spp. reported in non-passerine birds, such as doves, quails, and ducks (Fig. 3).

Four *Leucocytozoon* lineages were found in this study (Figs. 3 and 7). Using the PacBio protocol, a co-infection of *Haemoproteus* sp. hSTVAR01 and three different lineages of *Leucocytozoon* sp. (lBUVIR02/lBUVIR06/lSTOCC16) were detected in one *B. virginianus* (Additional file 2: Figure S1), and the phylogenetic relationship between them is shown in Figs. 3 and 7. The lineage hBUVIR02 is closely related to the lineages lBUBSUM01, lBUBCAP01, and lSTAL8 (Figs. 7 and 8; Additional file 1: Table S1C). The lineages lBUVIR06 and lSTOCC16 are closely related (genetic distance = 0.004 ± 0.003, Additional file 2: Figure S1) and clustered with *Leucocytozoon danilewskyi* (EU624137, lBUBO01 [44]) found in Eurasian Eagle-owl *Bubo bubo* (Figs. 3, 7, and 8; Additional file 1: Table S1C). The new *Leucocytozoon* lBUVIR10 is a distant lineage found in another *B. virginianus* in co-infection with *Haemoproteus* sp. hSTVAR01. This lineage shares a common ancestor with *Leucocytozoon* sp. lSTAL10 (ON932244, [9]) reported recently in a *S. uralensis* from Europe (Figs. 7 and 8). The genetic distance between lBUVIR10 and lSTAL10 was 0.02 ± 0.009.

Discussion

Owls are among the most charismatic groups of cosmopolitan non-passerine birds, including 229 extant species worldwide [45]. Unfortunately, knowledge of their haemosporidian parasites is still limited [3, 14–16]. Molecular records exist for only ten out of the 19 species found in North America, and only 42 out of the 229 known owl species [16], including the results of this study

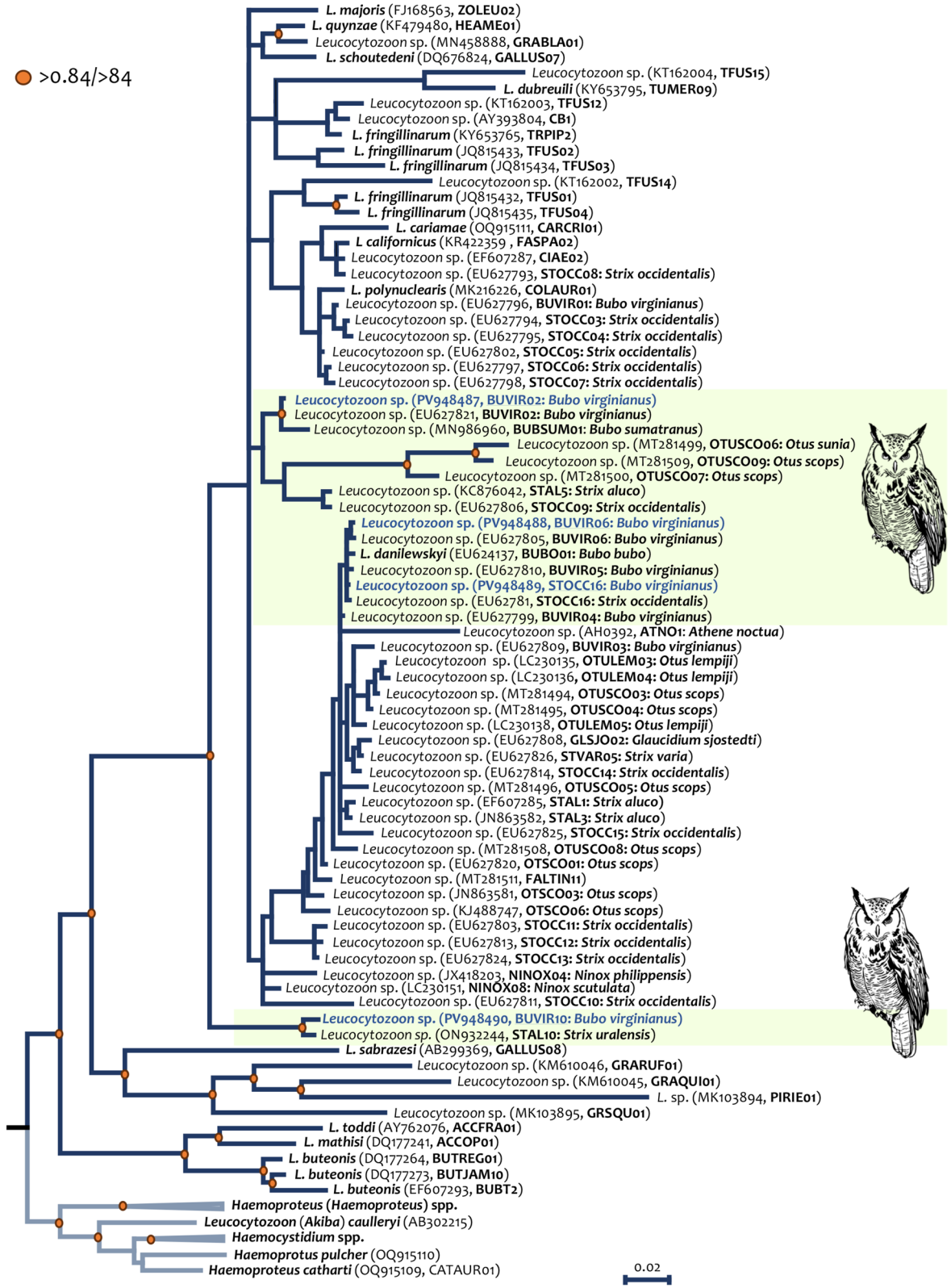


Fig. 7 Phylogenetic hypotheses of *Leucocytozoon* lineages based on partial *cytb* gene sequences (N = 87, 407 bp excluding gaps). Branch colors indicate different genera/hosts. GenBank accession numbers and MalAvi lineage for all parasite sequences used in this analysis are provided in parentheses. Lineages/species reported in this study are written in blue. Orange dots at the nodes mean a posterior probability greater than 0.84 and a bootstrap greater than 84

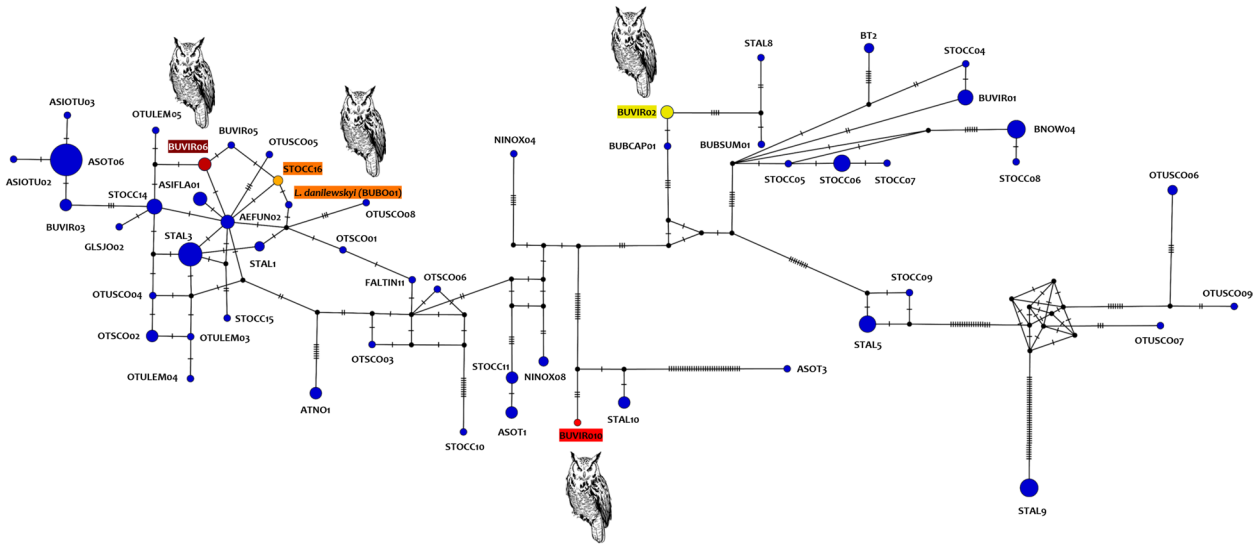


Fig. 8 Minimum spanning network for *Leucocytozoon* based on partial *cytb* gene lineages (N = 143, 400 bp excluding gaps) identified from owls. Circles are drawn in proportional to the frequency at which the lineages were detected. Lines between circles have the number of mutations that separate two lineages. *Leucocytozoon danilewskyi* (lBUBO01) and its closely related lineage lSTOCC16 found in this study are indicated in orange. Other lineages reported here are shown in different colors

(information summarized in additional file 1: Table S1B, D-E).

The lack of data can be explained, at least in part, by the fact that owls are primarily solitary and nocturnal birds, and they are also challenging to capture and handle. Additionally, most owl species are endangered, near threatened, and/or protected by law. Specifically, in the United States, all native owl species are protected by the Migratory Bird Treaty Act of 1918, as well as state laws. Therefore, taking advantage of rescued birds is a viable option for studying the frequency and biodiversity of haemosporidians and other symbionts in this vertebrate group. Despite the relatively few species investigated so far, the molecular data available confirm that owls worldwide are often infected by multiple lineages/species of haemosporidians in high prevalence (Additional file 1: Table S1B, S1E) [5, 6, 9, 12, 19, 39–44]. Consistent with previous observations, a high frequency and diversity of parasites were found circulating in these species, even considering our small sample size (Table 1).

At the time of this investigation, there were 262 haemosporidian *cytb* lineages reported for owls around the world: 101 *Haemoproteus*, 91 *Leucocytozoon*, and 70 *Plasmodium* lineages (Additional file 1: Table S1B, S1E), including those lineages found in this study. To our knowledge, the record of *Haemoproteus* sp. hMEGASI01 in *M. asio* (Additional file 1: Table S1B) is the first report based on molecular data for this species. A previous study found *Plasmodium* and *Haemoproteus* spp. in captive individuals using only microscopy [46]. The lineage hMEGASI01 shares a recent common ancestor with hSTOCC02 (Genetic distance = 0.002 ± 0.002) reported for the genus *Strix* from North America [16], suggesting that both may be part of a morphospecies circulating in the United States that has not been characterized yet. However, additional data (morphology and mt genomes) are required to describe this new putative parasite species.

The hSTVAR01 lineage, found in *B. virginianus* and *S. varia*, has been widely reported in the United States (Additional file 1: Table S1B, S1D-E), and it can be considered as a *H. syrnii* lineage (the closest to the hSTAL2 lineage). *Haemoproteus* (*Parahaemoproteus*) *syrnii* is one of the nine haemosporidian morphospecies described in owls (*Haemoproteus noctuae*, *Haemoproteus ilanpapernai* (*species inquirenda* [30]), *Plasmodium subpraecox*, *Plasmodium fallax*, *Plasmodium gundersi*, *Plasmodium hexamerium* (also in Turdidae), *P. elongatum* (also in Passeridae), and *L. danilewskyi*) [3, 14, 15]. It was first described in *S. aluco,* and similar morphotypes have been reported in more than 30 species from the Strigidae and Tytonidae families [3]. Consistently, it is one of the most common haemosporidians found in owls (Figs. 5 and 6) using molecular data when considering all the lineages assigned to this species (hSTAL2, hASIOTU05, hCULKIB01, and hSTVAR01). The hSTAL2 lineage corresponds to the redescription of the species made by Karadjian et al. [39], which has been reported in six European countries for at least four owl species, including the *S. aluco* (Additional file 1: Table S1B, S1E). On the

other hand, hSTVAR01 has been reported in 16 states of the United States in five owl species (Additional file 1: Table S1B and S1D-E). The genetic distance between these two *H. syrnii* lineages (*cytb* gene: 0.004 ± 0.003 and mtDNA: 0.006 ± 0.001) and their distribution suggest that they are likely a result of geographic population structure for this parasite, with one circulating in Europe (hSTAL2) and another in the United States (hSTVAR01). Indeed, all mt genome sequences obtained here for STVAR01 in both species of owls rescued from five states were 100% identical (Additional file 1: Table S1A).

It is worth noting that lineages hASIOTU05 and hCULKIB01, previously considered *H. syrnii*, are genetically distant from hSTAL2 and hSTVAR01 (genetic distances greater than 3% at the *cytb* fragment) and did not form a monophyletic group (Figs. 4 and 5), suggesting that these two lineages could belong to a different *Haemoproteus* species, calling for detail morphological analysis of blood stages. Although additional data are needed, it appears that using morphology and sequencing the small *cytb* fragments to characterize *Haemoproteus* species may lead to a polyphyletic taxon, at least in the case of *H. syrnii*. The limitations of morphology on separate species are commonly referred to as cryptic species [47]. However, the term typically applies to species that are morphologically indistinguishable but genetically distinct, forming monophyletic groups known as species complexes [47]. This is not the case here, as non-related lineages were perceived to have a similar morphology and classified as *H. syrnii*. This may be due to insufficient material for microscopic examination, technical limitations, lack of information from available morphological traits, presence of convergent morphological characteristics and/or a combination of these factors. It is worth mentioning that the morphology of volutin granules—these are roundish, prominent, and of various sizes in different parasite lineages—is considered a distinct character of parasites of the *H. syrnii* group. How parasites might be different based on this character is unknown, as detailed morphological analysis is absent for most of the *H. syrnii*-like lineages.

The closest lineage to *Haemoproteus* (*Parahaemoproteus*) *noctuae* (lineage hCIRCUM01, [41]) is hASIOTU05 found in *A. otus* (genetic distance 0.002 ± 0.002) (Figs. 4 and 5). This finding suggests that hASIOTU05 could be a lineage of *H. noctuae,* which was described in *A. noctua* and has been reported in more than 30 owl species (Figs. 4 and 5, Additional file 1: Table S1B, E). Unfortunately, a sample with a single infection with hASIOTU05 was unavailable, so only its partial *cytb* fragment was used in this investigation (Fig. 4). The other closely related lineage to *H. noctuae* hASIOTU05 is hASIOTU04 (genetic distance < 0.01), whose mitochondrial genome was included in this study for comparison (Fig. 3). All analyses performed here showed a recent common ancestor between them (Figs. 3, 4 and 5), confirming a previous finding [9].

Although the lineage hCULKIB01 (common in owls from Europe and Asia) possesses volutin granules, it seems to be unrelated to both *H. syrnii* or *H. noctuae*. It has been reported in *S. aluco* from Europe and shares a recent common ancestor with lineage hATHCUN01 found in Burrowing Owl *Athene cunicularia* from Brazil and with the *Haemoproteus* sp. hSTVAR06 found also here in *S. varia* (Fig. 5). Although Barino et al. [42] stated that these parasites found in Brazil were lineages of *H. syrnii*, Ilgūnas et al. [9] suggested that this parasite could be a new species given that the morphology of its gametocytes does not coincide with those of the *H. syrnii* description due to the lack of volutin granules [39], thus their descriptions and classification should be re-visited. Instead, the analysis of the genetic data suggests that lineages hSTAL4, hSTRURA02, hSTRURA03, and hSTVAR03 could be considered lineages of *H. syrnii* (Additional file 1: Table S1C).

Although owls frequently harbour co-infections (e.g., [9]), here only two *B. virginianus* had co-infections with *Haemoproteus* and *Leucocytozoon*, and one *M. asio* had a mixed infection with two *Plasmodium* species. Regarding *Leucocytozoon*, three of the four lineages detected here were found only in one individual using the PacBio protocol. Two of those lineages (lBUVIR06 and lSTOCC16) are closely related to *Leucocytozoon* (*Leucocytozoon*) *danilewskyi* Ziemann 1898 [3, 32] (lBUBO01 lineage: EU624137), the only *Leucocytozoon* morphospecies described in owls. This morphospecies was initially found in an *A. noctua* and has now been reported in at least 19 owl species, including *B. virginianus* [3]. The analysis of the molecular data suggests that lSTOCC16 may represent another lineage of this species [9]. Phylogenetic analyses and the haplotype network showed at least four to five very distant clades, suggesting an equal number of *Leucocytozoon* species, with *L. danilewskyi* just one of them. Indeed, given the genetic distance between the four lineages reported here, lBUVIR02 and lBUVIR10 should be considered as morphospecies distinct from the *L. danilewskyi* clade. *Leucocytozoon danilewskyi* has been suggested as being a complex of cryptic species with multiple lineages infecting different owl populations [32]. Indeed, the high genetic variation within the genus *Leucocytozoon* suggests that its taxonomy requires renewed attention as delimitation of morphospecies seems complex in parasites infecting almost all avian orders [3, 32]. Most of the time, *Leucocytozoon* species could not be linked to any parasite lineage recovered [32]. Indeed, describing *Leucocytozoon* species has

been challenging due to low parasitaemia (some of them can be only detected by PCR [48]), the frequent presence of co-infections, gametocyte deformation during blood smear preparation [32, 48], and the low number of informative characters in developing and mature gametocytes [3, 32]. It is worth mentioning that it is still unclear if *L. danilewskyi* gametocytes developing in roundish and fusiform host cells indeed belong to the same species [32]. Gametocytes in roundish host cells are common in owls and often coincide with gametocytes in fusiform host cells; both these gametocyte types are assumed to be *L. danilewskyi*, but they might belong to different species occurring in co-infections. Genetic proof is needed. The application of the long-read sequencing method might contribute to unravelling this issue. Furthermore, more attention is needed on the morphology of fusiform processors before concluding cryptic speciation in *Leucocytozoon* parasites; this character is taxonomically important, but remains insufficiently explored in taxonomy [32, 49].

To this point, some species are morphologically indistinguishable in their blood stages, as is the case with *Leucocytozoon.* In contrast, other morphospecies identified using erythrocytic stages may be polyphyletic, as was observed with *Haemoproteus*. Together, these findings suggest that a critical evaluation of the limited number of accessible characters in blood smears, along with a more detailed examination of these characters, is necessary. Thus, developing criteria for a molecular-based taxonomy in the absence of or with limited morphological evidence is a matter of some urgency for these parasites. In the remarkably genetically diverse *Leucocytozoon* parasite, this approach might be the only way to solve taxonomic problems at the species level.

Results from the *Plasmodium* spp. mixed infection (pPADOM11 and pWW3) indicated that pPADOM11 is a lineage of *P. elongatum.* The molecular evidence from the nearly complete mitochondrial genome (0.005 ± 0.001 genetic divergence to the lineage pGRW06, accession number KY653801, Fig. 3) and the morphological data are both consistent with *P. elongatum.* The lineage pPADOM11 has been documented in owls (Northern Sawwhet Owl *Aegolius acadicus* and *S. varia*) from North America [43]. It has been frequently reported in different passerine families from North and South America, with over 100 records submitted to the MalAvi database, and less frequently in non-passerines, such as Gaviiformes, Anseriformes, Strigiformes, and Piciformes. Given its broad distribution, correctly assigning pPADOM11 to a species is important. The lineage pWW3, on the other hand, may share a recent common ancestor with *Plasmodium lutzi* (pTFUS05, KC138226) based on specific morphological traits (e.g., large roundish gametocytes and pigment clustering) and molecular data. Although pPADOM11 and pWW3 were observed in a mixed infection in one individual, the combination of different methodologies and protocols allowed for the separation of these lineages, facilitating the association of molecular data with known morphological species (pPADOM11) or classification to the subgenus level (pWW3).

Summarizing, this study highlights the importance of sampling owls and other birds collected and/or rescued, as well as those taken to rehabilitation centres, and samples collected from hunter-harvested game birds, to study the diversity of haemosporidians. This approach could be considered for other non-passerine birds that are difficult to study in wildlife. Importantly, Next Generation Sequencing technologies generating long reads, such as the one implemented here (HmtG-PacBio HiFi protocol), allow for the characterization of co-infection and/or mixed infection, which predominate in wildlife. This study also linked genetic data (mt genome and *cytb* gene lineages) to *H. syrnii* and *P. elongatum*. These common parasites are now confirmed to infect different owl species in the United States. The analysis of available genetic data suggests that a more precise connection between morphospecies and lineages is necessary to prevent the proliferation of non-monophyletic species. It is worth noting that the lineage/species frequencies discussed here (e.g., haplotype networks) may be underestimated because only one sequence per lineage/species per study is generally submitted to the GenBank and/or MalAvi database (Additional file 1: Table S1E). A good practice is to submit all sequences obtained in each study, along with their corresponding metadata (e.g., precise location, time of collection, host species identification, and specimen number), so that overall frequencies can be accurately estimated. Although studying the exo-erythrocytic development of avian haemosporidian parasites was not part of the study's aims, birds from rehabilitation centres can be used to opportunistically study haemosporidian development, in vitro ex-flagellation, and ookinete development, as well as the pathological effects of these neglected avian infections.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05612-2.

Additional file 1.

Additional file 2.

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Author contributions
M. Andreína Pacheco: Conceived and designed the experiments, performed the experiments, nalysed the data, and wrote the first and final draft. Erica A. Miller: contributed reagents/samples/materials/analysis tools and corrected the final draft. Josef Harl: Contributed reagents/samples/materials/analysis tools and corrected the final draft. Francisco C. Ferreira: Analyzed the blood smears and corrected the final draft. Axl S. Cepeda: Run/maintain the HmtG-PacBio Pipeline and corrected the final draft. Gediminas Valkiūnas: Contributed reagents/samples/materials/nalysed the blood smears and corrected the final draft. Scott Beckerman: Contributed reagents/samples/materials/analysis tools and corrected the final draft. Mitchell Oswald: Contributed reagents/samples/materials/analysis tools and corrected the final draft. Nohra E. Mateus-Pinilla: Contributed reagents/samples/materials/analysis tools and corrected the final draft. Ananias A. Escalante: Conceived and designed the experiments, contributed reagents/samples/materials/analysis tools, and corrected the final draft.

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Data availability
All sequences generated and/or analyzed during the current study are available in the GenBank database under the following accession numbers: PV839541–PV839546 and PV948475-PV948515. The pipeline and database are available here: https://github.com/EscalanteLab/HmtG-PacBio-Pipeline.

Declarations

Competing interests
The authors declare no competing interests.

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