J. Parasitol., 96(1), 2010, pp. 170–177

IDENTIFICATION OF *LEUCOCYTOZOON TODDI* GROUP (HAEMOSPORIDA: LEUCOCYTOZOIDAE), WITH REMARKS ON THE SPECIES TAXONOMY OF LEUCOCYTOZOIDS

Gediminas Valkiūnas, Ravinder N. M. Sehgal*, Tatjana A. Iezhova, and Angus C. Hull†
Institute of Ecology, Vilnius University, Akademijos 2, Vilnius 21, LT-08412, Lithuania. *e-mail: gedvalk@ekoi.lt*

ABSTRACT: The current taxonomy of leucocytozoids (Haemosporida, Leucocytozoidae) is based on the morphology of blood stages of the parasites and on limited information about their specificity. Recent molecular studies have revealed a remarkable genetic diversity of leucocytozoids, indicating that their taxonomic diversity may be greater than in the current classifications. We addressed this issue using morphological data and phylogenetic analysis of the cytochrome *b* gene of 14 positively identified species of avian haemosporidians. Based on the current taxonomy, *Leucocytozoon toddi* is the sole species of leucocytozoids parasitizing falconiform birds. However, several distinct haplotypes have been revealed in falconiform birds, suggesting that *L. toddi* might be a group of cryptic species. We investigated the morphology of blood stages of leucocytozoids of 2 haplotypes (2 lineages from *Accipiter* spp. and 3 lineages from *Buteo* spp.) and concluded that these parasites can be readily distinguished due to length of the cytoplasmic processes of their host cells; therefore, they do represent distinct morphospecies. Morphology of the cytoplasmic processes of host cells warrants more attention in the taxonomy of *Leucocytozoon* species. *Leucocytozoon toddi* indeed is a species group that currently includes *Leucocytozoon mathisi*, *Leucocytozoon buteonis*, and *L. toddi*; additional morphospecies can be added to this group in the future. It is probable that some other morphospecies of *Leucocytozoon* also might be groups of closely related species.

Leucocytozoids (Haemosporida, Leucocytozoidae) are specific avian blood parasites which have been recorded in numerous species of birds in all continents except Antarctica (Bishop and Bennett, 1992). The majority of investigated leucocytozoids are transmitted by blood-sucking dipteran insects of the Simuliidae (Desser and Bennett, 1993; Valkiūnas, 2005; Forrester and Greiner, 2008). Many species of these parasites cause serious pathology in avian hosts, and some are responsible for lethal diseases in domestic and wild birds; therefore, they became objects of intensive investigation (Fallis et al., 1974; Desser and Bennett, 1993). However, virulence of the great majority of leucocytozoids for avian hosts and vectors remain insufficiently investigated. Limited evidence suggests that, being relatively non-pathogenic in naturally adapted hosts, leucocytozoids might cause diseases when they switch to new avian hosts (Garnham, 1966; Peirce et al., 1997; Valkiūnas, 2005; Forrester and Greiner, 2008). Due to the worldwide distribution of leucocytozoids, information on their diversity and pathogenicity is important for conservation purposes. This is particularly true for diurnal raptors of the Accipitridae, many species of which are endangered, are prevalently parasitized with *Leucocytozoon* spp. worldwide (Ashford et al., 1991; Valkiūnas, 2005), and suffer from *Leucocytozoon* spp. infections (Raidal and Jaensch, 2000; Tarello, 2006).

Based on limited information about blood stages (gametocytes and their host cells), 11 species of *Leucocytozoon* have been described in falconiform birds (Greiner and Kocan, 1977; Valkiūnas, 2005). Original descriptions of these species are incomplete, and the type material is absent for all of them. Gametocytes of the majority of these parasites develop in fusiform host cells, but gametocytes in roundish host cells have also been occasionally recorded (Fallis et al., 1974). Greiner and Kocan (1977) reviewed the taxonomy of leucocytozoids of falconiform birds. They concluded that only *Leucocytozoon toddi* Sambon, 1908 can be deemed as valid in this group of birds, based on the morphology of blood stages of the parasites. They also noted that *L. toddi* may, in fact, comprise a species complex, but left this as a subject for future research. Taxonomists widely accepted these conclusions and subsequently identified the parasite as *L. toddi* in all falconiform birds. This approach markedly simplified identification of species of leucocytozoids of falconiform birds, but the scale of diversity of these parasites remained unresolved.

Recent molecular investigations revealed remarkable genetic diversity of leucocytozoids (Hellgren, 2005; Martinsen et al., 2006; Sehgal, Hull et al., 2006; Ishak et al., 2008; Krone et al., 2008), indicating that the number of leucocytozoid species, and their taxonomic diversity, may be greater than is accepted in current classifications. For instance, phylogenetic analysis of the mitochondrial cytochrome *b* (cyt *b*) gene showed that lineages present in hawks of the genus *Buteo* are absent from hawks of the genus *Accipiter*; this is true in both sympatric and allopatric populations of these birds (Sehgal, Hull et al., 2006). Because there is over 10% sequence divergence between some lineages of these 2 haplotypes (Sehgal, Hull et al., 2006), these results suggested that *L. toddi* is likely a group of cryptic species, with different species or subspecies infecting *Buteo* spp. and *Accipiter* spp. This finding warrants further investigation because it can contribute new information about the diversification of leucocytozoids, which remain the least studied of the avian haemosporidian parasite groups (Valkiūnas, Iezhova, Loiseau, Smith et al., 2009).

The aims of the present study were to compare the morphology of gametocytes and host-cells of *L. toddi* of 2 different mitochondrial cyt *b* haplotypes in *Accipiter* spp. and *Buteo* spp. and to discuss possible interpretations of the taxonomy of leucocytozoids at the species level, in regard to recent molecular phylogenies.

MATERIAL AND METHODS

Study sites and blood samples

Blood samples were collected in California (37°40′N, 122°20′W), western Europe (the Curonian Spit in the Baltic Sea, 55°05′N, 20°44′E), and northern Kazakhstan (49°24′N, 46°48′E) during autumnal migration of birds from September to November 2003 and 2004, as described by Sehgal, Hull et al. (2006). Blood was taken by venipuncture of the medial metatarsal vein. Extraction of DNA and PCR and sequencing was done during our recent study (Sehgal, Hull et al., 2006). Blood smears were fixed in methanol and

Received 27 March 2009; revised 16 June 2009; accepted 6 August 2009.
* Department of Biology, San Francisco State University, 1600 Holloway Avenue, San Francisco, California 94132.
† Golden Gate Raptor Observatory, Building 1064, Fort Cronkhite, Sausalito, California 94965.
DOI: 10.1645/GE-2109.1

stained with Giemsa, as described by Valkiūnas (2005). Positive blood samples, as determined by PCR-based analysis (Sehgal, Hull et al., 2006), from 11 Cooper's hawks *Accipiter cooperii*, 4 northern sparrowhawks *Accipiter nisus*, 17 red-tailed hawks *Buteo jamaicensis*, 1 ferruginous hawk *Buteo regalis*, and 1 common buzzard *Buteo buteo* were used for investigations of the morphology of leucocytozoids and their host cells. The majority of samples were from California, except the northern sparrowhawk samples (1 sample from the Curonian Spit and 3 samples from Kazakhstan) and the common buzzard (1 sample from Kazakhstan). In morphological analysis, we used only a good-quality blood film, which is essential for taxonomic studies (Valkiūnas, Iezhova et al., 2008). Sehgal, Hull et al. (2006) described details of the study sites and collection of the material. The representative blood slides from all avian hosts were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania (accession nos. 47702–47714 NS).

Morphological analysis

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution, GmBH, Münster, Germany) was used to examine blood films, prepare illustrations, and to take measurements. Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1,000 red blood cells, or per 10,000 red blood cells if infections were light, i.e., <0.1%, as recommended by Godfrey et al. (1987). Blood films with intensity of *Leucocytozoon* spp. parasitemia ranging between 0.02% and 0.3% were used in this study.

Best-quality blood films from 2 individual birds of each species, except for the ferruginous hawk and the common buzzard (only 1 individual bird of each species), respectively, were used for morphometric analyses. Intensity of parasitemia in the investigated common buzzard was light (<0.01%), so this sample was not used for morphometric analysis. The morphometric features studied (Table I) were those defined by Valkiūnas (2005). A 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.

Phylogenetic analysis

The phylogenetic analysis was based on sequences (465 bp) of the cyt *b* gene from GenBank™. Because GenBank contains information about numerous, incorrectly identified species of haemosporidians (Valkiūnas, Atkinson et al., 2008), we used sequences of these parasites for which species were positively identified (for linkage of parasite lineages with their morphospecies, see Sehgal, Hull et al., 2006; Sehgal, Valkiūnas et al., 2006; Ishak et al., 2008; Perkins, 2008; Valkiūnas, Atkinson et al., 2008; Valkiūnas, Iezhova, Loiseau, Smith et al., 2009). Accession numbers of all sequences are shown in Figure 17. Phylogenetic analyses using maximum-parsimony techniques were conducted using PAUP*4.0b10 (Swofford, 2002). Searches used the bootstrap search option, with 1,000 stepwise addition replicates, using the TBR branch-swapping algorithm. In addition, we performed distance analyses using the Kimura 2-parameter distance model, and taxa were joined using neighbor-joining analysis. Simple consensus trees were constructed to summarize the results; both maximum-parsimony and neighbor-joining analyses resulted in trees with identical topologies. The sequence divergence between the different lineages (Table II) was calculated with the use of a Jukes-Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA 3.1 (Kumar et al., 2004).

RESULTS

Morphological analysis

There were no significant differences in all morphometric features of *Leucocytozoon* sp. gametocytes and their host cells in different individual hosts belonging to the same species of birds ($P > 0.05$ for all features studied; the data are not presented), so we pooled morphometric data for parasite measurements in different individual hosts belonging to the same bird species. These data are given in Table I.

The measurements of (1) macro- and microgametocytes, (2) nuclei of parasites, (3) nuclei of host cells, (4) the width of the cytoplasmic processes, and (5) the width of host-cell parasite complex overlap markedly between species in *Accipiter* and *Buteo* (Table I). Since the overlap is so great, it is hardly possible to attach much taxonomic significance to these features. This is particularly true because the great majority of *Leucocytozoon* infections are light in falconiform birds (<0.01%), frequently with just a few gametocytes present in blood films. Such samples usually are inappropriate for morphometric statistical analysis because of the small number of parasites available for measurements.

Length of the cytoplasmic processes of host cells, and some features that depend on this character (area of the cytoplasmic processes, length, and area of the host-cell parasite complex), markedly overlap in parasites developing in different species of birds belonging to the same genus (Table I). However, these features do not overlap, or only slightly overlap, in birds belonging to different genera. In other words, parasites from both species of *Accipiter* can be readily distinguished from parasites of both *Buteo* spp. due to the length of the cytoplasmic processes of their host cells and to the length and area of the host-cell parasite complex (see Table I; compare Figs. 1–8 and 9–16). In *Accipiter* spp., the values for these 3 features are significantly less than in *Buteo* spp. ($P < 0.001$ for comparisons of all these features between 2 *Accipiter* spp. and 2 *Buteo* spp.). Thus, primarily due to length of the cytoplasmic processes, leucocytozoids from *Accipiter* spp. can be readily distinguished from *Buteo* spp. This is true both for macro- and microgametocytes of these parasites (Table I).

Because of light parasitemia, only 7 non-deformed gametocytes (5 macro- and 2 microgametocytes) were seen and measured in the single common buzzard. Due to the small sample size, morphometric data from this bird are not shown in Table I. However, all morphometric features of parasites from the common buzzard overlap completely with the morphometry of leucocytozoids in the red-tailed hawk.

Phylogenetic analysis

Parasites from *Accipiter* spp. and *Buteo* spp. cluster together, but form distinct sub-clades in the phylogenetic tree (Fig. 17, clade a). Genetic divergence in cyt *b* gene between lineages from *Accipiter* spp. is 4.7% and ranges between 1.5% and 3.4% between different lineages from *Buteo* spp. (Table II). Genetic divergence between 2 lineages from *Accipiter* spp. and 3 lineages of *Buteo* spp. ranges between 7.1% and 10.1%.

Sequence divergence between all positively identified and morphologically readily distinguishable species of leucocytozoids ranges between 5.8% and 20.9% (Table II).

Parasites of *Accipiter* spp. and *Buteo* spp. are redescribed here as valid species.

REDESCRIPTION

Leucocytozoon mathisi França, 1912
(Figs. 1–8, Tables I, II)

Macrogametocytes (Figs. 1, 2, 5, 6): Develop in fusiform host cells. Gametocytes in roundish host cells have not been reported. Mode of growth, morphology of gametocytes, and their host cells indistinguishable from same stages of *L. toddi* from northern sparrowhawk (described by

TABLE I. Morphometry of mature gametocytes of *Leucocytozoon* spp. and their fusiform host cells in different avian hosts.

	Measurements (μm)*			
	Leucocytozoon mathisi		*Leucocytozoon buteonis*	
Feature	*Accipiter cooperii*	*Accipiter nisus*	*Buteo jamaicensis*	*Buteo regalis*
Macrogametocyte	n = 40	n = 30	n = 44	n = 44
Length	14.3–27.7 (19.6 ± 3.1)	14.8–26.3 (20.1 ± 3.1)	10.2–25.8 (19.4 ± 2.3)	12.6–29.3 (20.7 ± 3.8)
Width	2.4–11.4 (7.3 ± 2.0)	2.9–9.2 (5.6 ± 1.9)	5.2–10.5 (7.7 ± 1.4)	3.4–12.3 (7.3 ± 2.3)
Area	112.4–177.8 (144.3 ± 13.1)	89.6–150.7 (127.7 ± 15.6)	124.5–177.7 (151.7 ± 10.2)	114.8–206.1 (159.2 ± 20.8)
Parasite nucleus				
Length	2.0–4.7 (3.0 ± 0.7)	2.1–4.7 (3.0 ± 0.7)	2.0–5.3 (3.0 ± 0.7)	1.9–5.1 (3.1 ± 0.7)
Width	2.9–7.3 (4.6 ± 0.9)	2.5–5.7 (3.7 ± 0.8)	2.4–7.0 (4.8 ± 1.0)	3.1–8.0 (5.1 ± 1.2)
Area	5.3–16.7 (11.5 ± 2.4)	3.6–15.9 (9.4 ± 2.3)	5.5–16.2 (11.7 ± 2.5)	8.6–18.9 (12.6 ± 2.1)
Host cell nucleus				
Length	8.8–15.3 (11.5 ± 1.4)	6.8–12.0 (9.7 ± 1.2)	7.6–16.8 (12.7 ± 1.6)	7.8–16.5 (11.2 ± 1.8)
Width	1.1–4.4 (2.6 ± 0.7)	1.8–4.1 (3.1 ± 0.6)	1.5–3.7 (2.6 ± 0.5)	1.6–4.3 (2.8 ± 0.6)
Area	15.2–36.1 (24.3 ± 5.7)	14.5–32.0 (23.9 ± 5.3)	20.2–35.4 (27.6 ± 3.4)	11.7–36.7 (25.8 ± 5.8)
Cytoplasmic processes				
Length	1.9–13.7 (7.5±2.8)	3.6–10.9 (8.1±1.9)	12.7–30.5 (19.7±3.9)	12.1–26.8 (18.9±3.9)
Width	1.7–9.9 (4.1±1.7)	2.0–6.8 (4.0±1.9)	2.0–5.7 (3.5±0.8)	2.2–8.4 (3.7±1.3)
Area	6.6–25.1 (14.4±4.9)	5.4–28.7 (16.7±5.0)	21.1–45.8 (32.7±6.3)	16.5–44.0 (30.9±5.9)
Host-cell parasite complex				
Length	18.8–43.3 (33.8±6.5)	20.2–46.2 (36.9±6.8)	42.4–76.1 (58.9±8.1)	38.7–70.9 (57.9±8.2)
Width	6.9–14.2 (9.9±2.0)	4.9–13.2 (8.5±2.3)	6.9–14.7 (10.4±2.1)	6.4–15.0 (10.2±2.2)
Area	139.4–218.9 (181.0±19.8)	131.0–186.6 (168.9±14.6)	190.3–305.7 (236.9±25.4)	190.4–279.6 (232.0±23.9)
Microgametocyte	n = 18	n = 26	n = 23	n = 35
Length	16.3–21.6 (19.3 ± 1.7)	11.6–23.5 (15.2 ± 3.7)	17.7–28.1 (22.0 ± 2.5)	15.1–28.8 (21.9 ± 4.2)
Width	2.4–7.5 (4.4 ± 1.6)	2.1–8.3 (5.7 ± 1.9)	2.5–7.3 (5.1 ± 1.3)	2.1–9.9 (5.5 ± 2.4)
Area	91.7–139.5 (112.1 ± 14.2)	75.1–115.8 (95.0 ± 10.1)	107.8–156.2 (137.4 ± 11.1)	118.1–164.5 (137.3 ± 10.6)
Parasite nucleus				
Length	5.2–18.0 (11.4 ± 3.2)	3.4–18.1 (9.9 ± 4.1)	7.9–21.1 (13.3 ± 3.9)	5.4–24.9 (14.5 ± 5.9)
Width	1.5–7.1 (3.5 ± 1.8)	2.0–7.5 (4.8 ± 1.6)	2.6–6.6 (4.6 ± 1.2)	1.9–9.5 (5.0 ± 2.0)
Area	22.5–76.7 (40.1 ± 13.0)	20.1–100.0 (42.8 ± 14.3)	38.9–88.5 (60.8 ± 14.6)	28.1–106.4 (64.7 ± 17.6)
Host cell nucleus				
Length	8.9–15.6 (10.9 ± 1.5)	7.0–11.7 (9.4 ± 1.5)	9.4–19.1 (12.9 ± 2.0)	6.3–15.4 (12.5 ± 2.0)
Width	2.5–4.1 (3.1 ± 0.5)	2.2–4.1 (3.2 ± 0.5)	1.7–4.2 (3.1 ± 0.6)	1.8–3.8 (3.2 ± 1.9)
Area	21.7–32.6 (27.2 ± 3.6)	17.0–31.0 (23.3 ± 3.6)	25.9–37.5 (31.5 ± 3.1)	20.9–40.3 (30.7 ± 5.0)
Cytoplasmic processes				
Length	1.9–10.1 (6.3 ± 2.3)	1.8–9.3 (6.0 ± 1.9)	15.3–24.7 (19.1 ± 2.4)	12.8–22.3 (17.6 ± 2.8)
Width	2.1–7.7 (4.5 ± 1.5)	2.8–6.8 (4.4 ± 1.2)	2.3–5.9 (3.8 ± 0.9)	2.0–6.3 (3.6 ± 1.1)
Area	7.8–19.5 (13.5 ± 4.0)	6.2–18.7 (11.7 ± 2.9)	18.1–42.5 (31.4 ± 5.7)	16.7–42.9 (28.0 ± 7.1)
Host-cell parasite complex				
Length	16.8–37.3 (27.8 ± 5.8)	15.2–38.7 (26.5 ± 6.4)	45.8–67.2 (56.7 ± 5.6)	38.2–68.5 (55.2 ± 7.1)
Width	6.4–11.4 (9.0 ± 1.4)	5.6–11.7 (8.7 ± 1.8)	7.1–14.7 (10.2 ± 2.4)	6.0–13.0 (8.6 ± 2.2)
Area	104.0–160.2 (141.2 ± 14.7)	103.4–150.8 (127.5 ± 12.5)	183.4–263.7 (219.9 ± 21.8)	180.5–247.0 (204.3 ± 16.0)

* Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

Valkiūnas, 2005). Average length of cytoplasmic processes of host cells in type vertebrate host and Cooper's hawk close to 8 μm (Table I).

Microgametocytes (Figs. 3, 4, 7, 8): General configuration as for macrogametocytes, with usual haemosporidian sexually dimorphic characters. Average length of cytoplasmic processes of host cells in type vertebrate host and Cooper's hawk close to 6 μm (Table I).

Taxonomic summary

Type host: Accipiter nisus (Falconiformes, Accipitridae).
Additional host: Accipiter cooperii.

DNA sequences: Mitochondrial cyt *b* lineages with GenBank accession DQ177252 and DQ177250.

Type locality: Collares, Portugal; according to original designation by França (1912).

Site of infection: Erythrocytes; no other data.

Prevalence and distribution: During autumnal migration, the overall prevalence of *L. mathisi* infection in the type host is over 30% in Europe (Valkiūnas, 2005) and is approximately 50% in the Cooper's hawk in California (Sehgal, Hull et al., 2006). This parasite is widespread in *Accipiter* spp. in the Holarctic. It is worth noting that the same parasite lineage has been recorded in the northern sparrowhawks in western Europe and in

TABLE II. The sequence divergence (in percentage) between mitochondrial cytochrome *b* lineages of positively identified species of *Leucocytozoon* and *Plasmodium*.

Species*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *P. ashfordi*	0													
2. *P. lucens*	8.2	0												
3. *L. schoutedeni*	14.9	15.1	0											
4. *L. macleani*	15.9	16.2	7.1	0										
5. *L. majoris*	16.6	16.4	7.1	8.0	0									
6. *L. danilewskyi*	15.9	15.3	6.9	9.3	7.1	0								
7. *L. danilewskyi*	16.4	15.7	7.3	9.1	7.1	0.4	0							
8. *L. fringillinarum*	15.7	14.7	5.8	6.9	5.8	6.7	6.7	0						
9. *L.* sp.	16.2	14.4	6.5	7.5	6.7	7.5	7.1	2.2	0					
10. ***L. buteonis***	15.9	15.5	16.6	19.2	17.7	18.1	18.5	17.7	19.4	0				
11. ***L. buteonis***	18.5	17.0	17.5	19.8	18.8	19.0	19.4	19.2	20.9	3.0	0			
12. ***L. buteonis***	16.6	15.3	16.4	18.3	18.1	18.1	18.5	17.2	19.0	1.5	3.4	0		
13. ***L. mathisi***	17.5	16.6	17.5	17.5	19.4	19.8	19.8	18.1	18.5	8.8	10.1	8.8	0	
14. ***L. mathisi***	16.6	15.9	16.6	17.9	17.7	18.0	19.2	17.9	18.5	7.1	8.4	7.1	4.7	0

* The species are numbered as in Figure 17, in which GenBank accession numbers of their lineages are given. The sequence divergence was calculated with the use of a Jukes–Cantor model of substitutions. The names of species of the *Leucocytozoon toddi* group are given in bold.

northern Kazakhstan (Sehgal, Hull et al., 2006). The presence of *L. mathisi* in other falconiform birds, particularly outside the Holarctic, should be investigated by combining morphological and DNA sequence data.

Specimens: Type material is absent; it should be designated from the type vertebrate host from the type locality. Voucher specimens (*A. nisus*, 2004, Curonian Spit, collected by G. Valkiūnas, and *A. cooperii*, 2004, California, collected by A. Hull) were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania and in the Queensland Museum, Queensland, Australia (accession nos. 47702–47706 NS, 47707–47710 NS, and G465249, G465248), respectively.

Remarks

The original description of *L. mathisi* is incomplete and lacking both illustrations and morphometric data (França, 1912). However, the presence of conical-form cytoplasmic processes was noted in the description of this parasite, which was originally found in the northern sparrowhawk. Because the presence of short, frequently conical-form cytoplasmic processes (Figs. 1, 3, 5, 7) is the main morphological character of leucocytozoids of the northern sparrowhawk, validation of the species name *L. mathisi* is possible. This name can be used for closely related lineages of leucocytozoids with short cytoplasmic processes of their host cells (Figs. 1–8, 17; Table I).

Leucocytozoon mathisi can be readily distinguished from *L. buteonis*; host cells harboring *L. mathisi* possess cytoplasmic processes that are more than twice as short when compared with cells that are infected with gametocytes of *L. buteonis* (compare Figs. 1–8 and 9–16). Cytoplasmic processes longer than 15 μm in length were not seen in *L. mathisi* infections, but they predominated with *L. buteonis* (Table I).

Leucocytozoon buteonis (Coatney and Roudabush, 1937) emend. Valkiūnas, 2005
(Figs. 9–16, Tables I, II)

Macrogametocytes (Figs. 9, 10, 13, 14): Develop in fusiform host cells. Gametocytes in roundish host cells not previously recorded. Mode of growth and morphology of gametocytes similar to same stages of *L. toddi* as described by Valkiūnas (2005). Average length of cytoplasmic processes of host cells in all investigated vertebrate hosts close to 20 μm (Table I).

Microgametocytes (Figs. 11, 12, 15, 16): General configuration as for macrogametocytes, with usual haemosporidian sexually dimorphic characters. Average length of cytoplasmic processes of host cells in all investigated vertebrate hosts close to 17 μm (Table I).

Taxonomic summary

Type host: Buteo jamaicensis (Falconiformes, Accipitridae).
Additional hosts: Buteo buteo, *B. regalis*.

DNA sequences: Mitochondrial cyt *b* lineages with GenBank accession DQ177273, DQ177253, and DQ177264.

Type locality: Peru, Nebraska, according to the original designation by Coatney and Roudabush (1937).

Prevalence and distribution: During autumnal migration in California, 26% of the red-tailed hawks were infected (Sehgal, Hull et al., 2006). This parasite has been frequently recorded in Europe and Kazakhstan (G. Valkiūnas, pers. obs.), so is widespread in the Holarctic. Presence of *L. buteonis* in other falconiform birds, particularly outside the Holarctic, should be investigated by combining morphological and DNA sequence data.

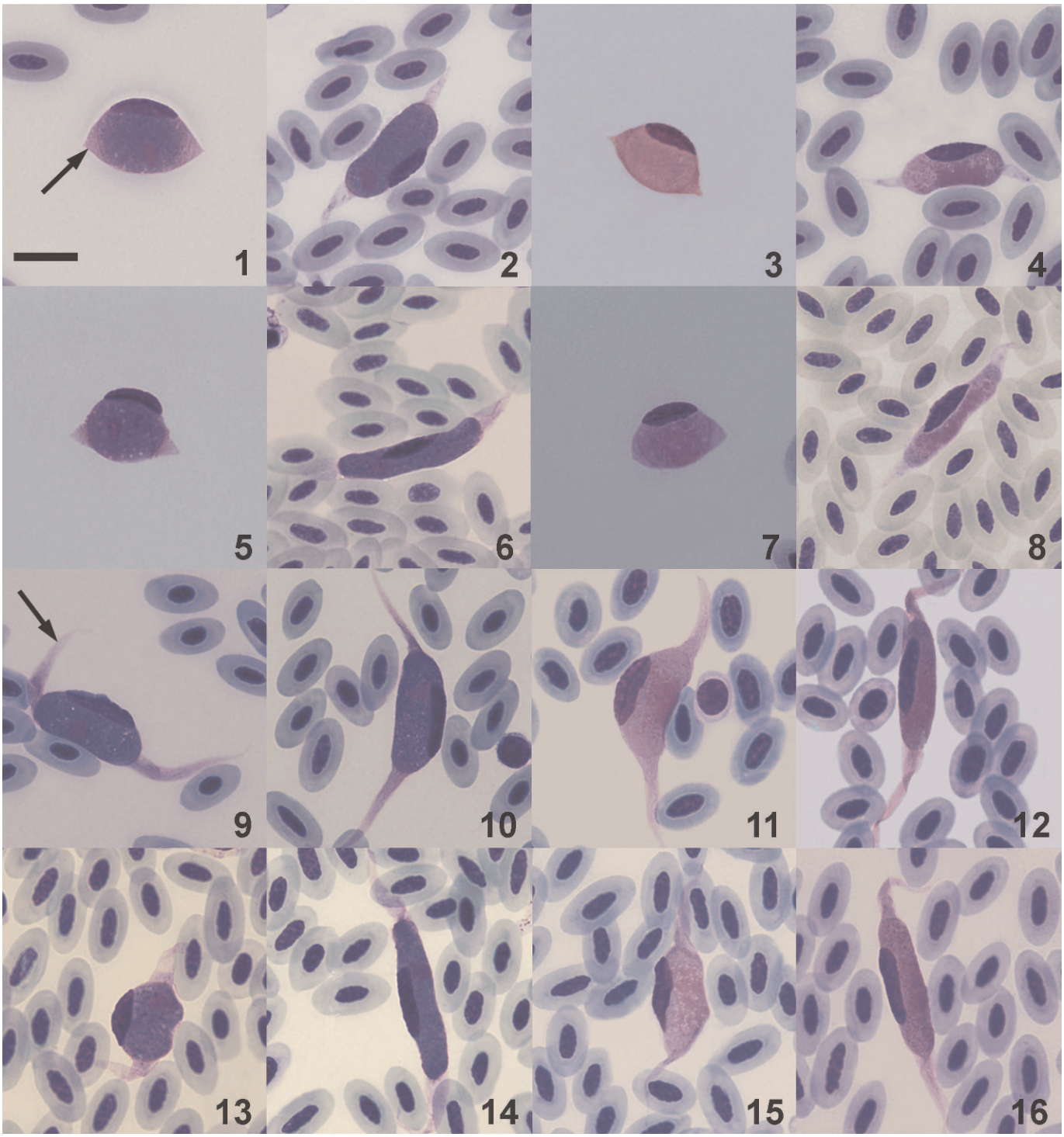
Specimens: Type material is absent; it should be designated from the type vertebrate host from the type locality. Voucher specimens (*B. jamaicensis*, 2003, California, collected by A. C. Hull) were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania and in the Queensland Museum, Queensland, Australia (accession nos. 47711–47713 NS, and G465250), respectively.

Remarks

The original description of *L. buteonis* (Coatney and Roudabush, 1937) is accompanied both by illustrations and morphometrics of the parasite, whose main features correspond well to the characters reported during this study (Figs. 9–16, Table I). This description provides an opportunity to validate the species name *L. buteonis*. This name can be used for closely related lineages of leucocytozoids with long cytoplasmic processes of their host cells (Figs. 9–16, 17, Table I).

DISCUSSION

Species of *Leucocytozoon* from 5 species of diurnal raptors (Fig. 17, clade a) have the main diagnostic characters of morphospecies *L. toddi* (Greiner and Kocan, 1977; Valkiūnas, 2005). They develop in fusiform host cells; nuclei of the host cells are cap-like or almond-shaped, or by their shape resemble the nuclei of uninfected erythrocytes. The nuclei extend less than 1/3 of the circumference of the gametocytes (Figs. 1–16, Table I). Due to these features, the parasites from species of *Buteo* and *Accipiter* were attributed to *L. toddi* by Sehgal, Hull et al. (2006). Because genetic divergence among lineages of *L. toddi* in different species of Accipitridae is large (Table II), these authors concluded that *L. toddi* is most likely a group of cryptic species, with different species or subspecies infecting *Buteo* spp. and *Accipiter* spp. The present study shows that parasites of *Accipiter* spp. and *Buteo* spp. can be readily distinguished due to the length of the



FIGURES 1–16. Gametocytes of *Leucocytozoon mathisi* (**1–8**) and *Leucocytozoon buteonis* (**9–16**) in fusiform host cells from the blood of *Accipiter cooperii* (**1–4**), *Accipiter nisus* (**5–8**), *Buteo jamaicensis* (**9–12**), and *Buteo regalis* (**13–16**). Macrogametocytes (**1, 2, 5, 6, 9, 10, 13, 14**). Microgametocytes (**3, 4, 7, 8, 11, 12, 15, 16**). Arrows: fusiform cytoplasmic processes of host cells. Giemsa-stained thin blood films. Bar = 10 μm.

cytoplasmic processes of their host cells and other features associated with length of the processes (compare Figs. 1–8 and 9–16, Table I). Due to these morphological characters, and also to clear genetic differences (see Fig. 17, Table II), we recommend considering the leucocytozoids from *Accipiter* and *Buteo* birds as distinct species.

Numerous synonyms of *L. toddi* and other *Leucocytozoon* spp. are available. They were discussed by Greiner and Kocan (1977) and Valkiūnas (2005) and represent a reserve for the taxonomic nomenclature of the leucocytozoids. The main diagnostic characters of *Leucocytozoon* parasites from *Accipiter* and *Buteo* species match the descriptions of *L. mathisi* and *L. buteonis*,

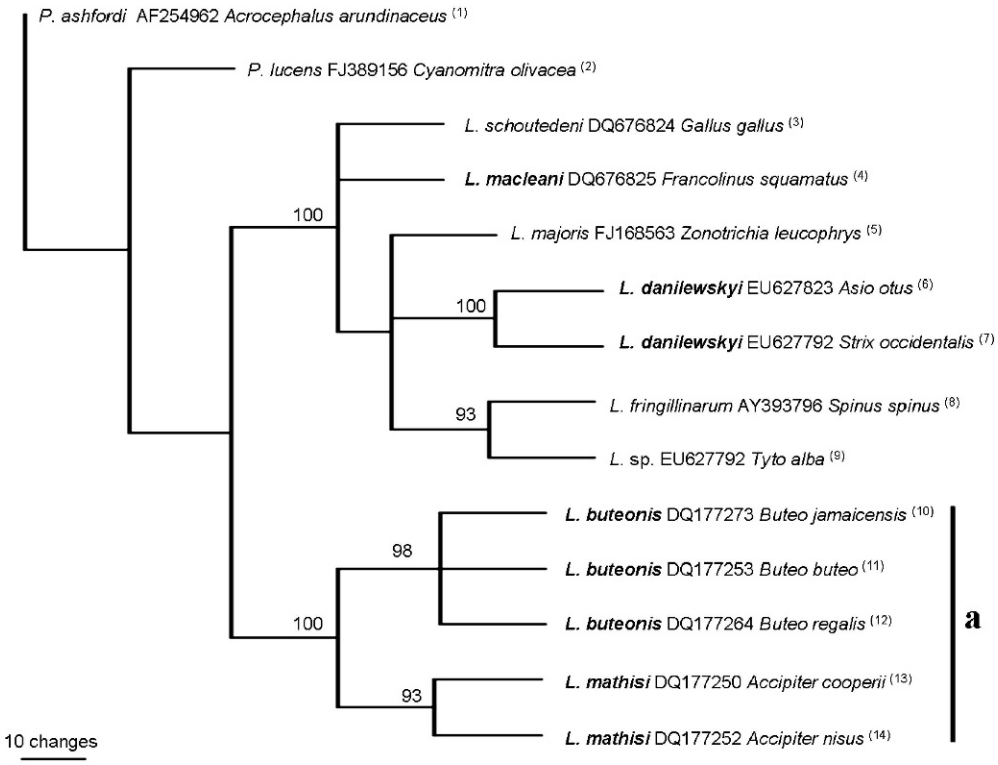


FIGURE 17. Maximum-parsimony phylogeny of 12 mitochondrial cytochrome *b* lineages of *Leucocytozoon* species. Lineages of 2 species of avian *Plasmodium* were used as an outgroup. GenBank accession numbers of the sequences, and the Latin names of birds, are given after the parasite species names. The lineages are numbered (ciphers in parentheses) as in Table II. Vertical bar (**a**) indicates a group of closely related lineages of the *Leucocytozoon toddi* group. The names of *Leucocytozoon* species, which develop in fusiform host cells, are given in bold. The branch lengths are drawn proportionally to the amount of change.

respectively, so we suggest the validation of these names and use them to identify morphologically similar parasites (Figs. 1–16) of closely related lineages (Fig. 17, clade a). This conclusion is in accord with the hypothesis of Greiner and Kocan (1977) that *L. toddi* might represent morphologically similar taxa comprising a species complex. We agree, and suggest identifying parasites of this complex as a group of *L. toddi* species. According to the current knowledge, this group of species includes *L. mathisi*, *L. buteonis*, and *L. toddi*. The certain number of species in the *L. toddi* group remains unclear; it should be defined by a close linking of genetic and morphological data.

The present study shows that morphology of the cytoplasmic processes of host cells warrants more attention in the taxonomy of *Leucocytozoon* species. Detailed analysis of morphology of the processes has not been applied in taxonomy of these parasites, so far. It is worth noting that development of the fusiform processes of host cells is induced by developing gametocytes. It is possible that compactly packed bundles of microtubules, which develop in gametocytes and are situated parallel to the long axis of the parasites, take part in formation of the cytoplasmic processes (Desser et al., 1970; Steele and Noblet, 1993). Because the development of the fusiform processes is parasite species-specific (Valkiūnas, 2005), it should be genetically determined and might reflect evolutionary adaptations and, thus, be applied in *Leucocytozoon* taxonomy at the species level. We encourage using morphological analysis of the cytoplasmic processes during description of *Leucocytozoon* spp. Such processes develop only in some species of *Leucocytozoon*; the function of the processes remains unknown.

It is important to note that the character presence–absence of the cytoplasmic processes can hardly be applied in the taxonomy of leucocytozoids, on either the level of genera or subgenera, because lineages of parasites possessing the cytoplasmic processes (*Leucocytozoon danilewskyi*, *L. buteonis*, *Leucocytozoon macleani*, and *L. mathisi*) are paraphyletic in the phylogenetic analysis (Fig. 17). This conclusion is in accord with former taxonomic conclusions which are based on morphology and life histories of leucocytozoids (Fallis et al., 1974).

Gametocytes in roundish host cells, which lack the cytoplasmic processes, were not seen during this study. They are rarely recorded in *L. toddi* (Greiner and Kocan, 1977). The reasons for the paucity of gametocytes in roundish host cells remain unclear (Valkiūnas, 2005). The following explanation for this finding seems most probable. First, gametocytes in roundish host cells develop in species of the *L. toddi* group only at the beginning of primary parasitemia, and the time of their existence is short, so they might be difficult to record, particularly during seasonal migration and wintering of birds when the majority of samples are most frequently collected, but infections are light or latent. They also might not develop in all avian hosts, as is a case with gametocytes in fusiform host cells of *Leucocytozoon simondi* (Desser et al., 1978; Desser and Bennett, 1993). Second, gametocytes in roundish host cells may not be parasites of the *L. toddi* group, but rather rare cases of co-infection with different species of *Leucocytozoon*. Additional investigations using DNA sequence data are needed to deal with this issue. However, before proven otherwise, we suggest attributing the rare records of

gametocytes in roundish host cells to parasites of the *L. toddi* group, as was justified by Greiner and Kocan (1977).

The identification of species of the *L. toddi* group might be used as an example for a possible development of the current taxonomy of *Leucocytozoon* at the species level (see Fallis et al., 1974; Valkiūnas, 2005; Forrester and Greiner, 2008). Due to the large genetic divergence of *Leucocytozoon* spp. (Hellgren, 2005; Sehgal, Hull et al., 2006; Ishak et al., 2008), it is probable that some morphospecies of *Leucocytozoon* are, in fact, groups of closely related species or subspecies; this might be distinguished using molecular markers as is the case with *L. buteonis* and *L. mathisi*. Importantly, these markers might result in new, valuable, morphological criteria for the development of species taxonomy of haemosporidians, as was the case during this and other recent studies (Valkiūnas et al., 2007). Such criteria are important because identification of *Leucocytozoon* morphospecies is currently based primarily on a limited number of morphological characters of their host cells, but not of the parasites (Valkiūnas, 2005; Forrester and Greiner, 2008), so the species taxonomy of leucocytozoids certainly needs further development.

It is worth noting that 5 lineages of the *L. toddi* group are monophyletic in our present cyt *b* gene phylogenetic analysis (Fig. 17), with intraspecific genetic divergence between lineages of *L. mathisi* and *L. buteonis* of <5% (Table II). Interestingly, the genetic divergence is >5% between morphologically readily distinguishable *L. mathisi*, *L. buteonis*, *Leucocytozoon schoutedeni*, *L. danilewskyi*, and *Leucocytozoon fringillinarum*. It is probable that an approximate difference of <5% in the cyt *b* gene reflects intraspecific variation of *Leucocytozoon* spp., and a difference of >5% indicates an interspecific level of divergence of these parasites. These data are in accord with Hellgren et al. (2007) and Valkiūnas, Iezhova, Loiseau, Smith et al. (2009), who concluded that avian *Haemoproteus* and *Plasmodium* species with a genetic differentiation of over 5% in mitochondrial cyt *b* gene are expected to be morphologically differentiated. It is probable that the criterion of a genetic difference of >5% in cyt *b* gene reflects interspecific divergence in many groups of avian haemosporidians, and so can be used for better understanding of phylogenetic trees based on this gene. It should be noted, however, that genetic divergence in the cyt *b* gene between some readily distinguishable morphospecies of avian haemosporidian parasites is <5%; it might be as low as 1% between some readily distinguishable morphospecies (Hellgren et al., 2007; Valkiūnas, Iezhova, Loiseau, Smith et al., 2009). Thus, the molecular criterion of >5% sequence divergence in the cyt *b* gene for identification of haemosporidian species should be developed and applied carefully, preferably by linking molecular and microscopical data. To fully accept this criterion, additional information about genetic distances between lineages of positively identified species of haemosporidians is needed. GenBank should only be used prudently for this purpose, because it hosts genetic sequences from numerous misidentified haemosporidian parasites (Valkiūnas, Atkinson et al., 2008). Accumulation of information on this subject would form a basis for the interpretation of cyt *b* gene phylogenetic trees of avian haemosporidians and in the taxonomy of these parasites using molecular data.

Genetic divergence in the cyt *b* gene, between the majority of positively identified species of *Leucocytozoon*, is >14% (Table II). Such great variation in this gene has not been recorded between species in any genus of haemosporidian parasites (Ricklefs et al., 2004; Hellgren et al., 2007; Perkins et al., 2007; Valkiūnas, Iezhova, Loiseau, Smith et al., 2009). This suggests rapid evolution of the cyt *b* gene in *Leucocytozoon* spp. in comparison to other genera of haemosporidians. According to the current classifications, the species diversity of *Leucocytozoon* is lowest in comparison to species of *Haemoproteus* and *Plasmodium* (Fallis et al., 1974; Bensch et al., 2004; Valkiūnas, 2005). This contradicts the remarkable genetic diversity of leucocytozoids. Further taxonomic investigations are required, preferably by combining molecular and phenotypic markers.

It is important to note that PCR detects very small numbers of unknown sporozoites in the peripheral circulation (Valkiūnas, Iezhova, Loiseau, and Sehgal, 2009), so detection of lineages of hemosporidians in vertebrate hosts should be carefully considered in ecological and evolutionary biology studies. To be accepted as the lineages of successfully developing species of hemosporidians, such PCR-based information should be supported with the detection of blood stages of the parasites. We thus emphasize an urgent need for the synthesis of information provided by tools of traditional parasitology and molecular biology in studies of haemosporidians, particularly in wildlife.

ACKNOWLEDGMENTS

This article benefited from comments made by Gerald W. Esch, Susan L. Perkins, and 2 anonymous reviewers. We wish to thank Dalia Kuveikytė for help in measuring parasites. The present study was supported by the Lithuanian State Science and Studies Foundation and by San Francisco State University.

LITERATURE CITED

Ashford, R. W., E. E. Green, P. R. Holmes, and A. J. Lucas. 1991. *Leucocytozoon toddi* in British sparrowhawks *Accipiter nisus*: Patterns of infection in nestlings. Journal of Natural History **25:** 269–277.

Bensch, S., J. Pérez-Tris, J. Waldenström, and O. Hellgren. 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? Evolution **58:** 1617–1621.

Bishop, M. A., and G. F. Bennett. 1992. Host-parasite catalogue of the avian haematozoa: Supplement 1; and Bibliography of the avian blood-inhabiting haematozoa: Supplement 2. Occasional Papers in Biology, Memorial University of Newfoundland **15:** 1–244.

Coatney, G. R., and R. L. Roudabush. 1937. Some blood parasites from Nebraska birds. American Midland Naturalist **18:** 1005–1030.

Desser, S. S., J. R. Baker, and P. Lake. 1970. The fine structure of *Leucocytozoon simondi*. I. Gametogenesis. Canadian Journal of Zoology **48:** 331–336.

———, and G. F. Bennett. 1993. The genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. *In* Parasitic protozoa, vol. 4, 2nd ed., J. P. Kreier and J. R. Baker (eds.). Academic Press, New York, New York, p. 273–307.

———, J. Stuht, and A. M. Fallis. 1978. Leucocytozoonosis in Canada geese in Upper Michigan. I. Strain differences among geese from different localities. Journal of Wildlife Diseases **14:** 124–131.

Fallis, A. M., S. S. Desser, and R. A. Khan. 1974. On species of *Leucocytozoon*. Advances in Parasitology **12:** 1–67.

Forrester, D. J., and E. C. Greiner. 2008. Leucocytozoonosis. *In* Parasitic diseases of wild birds, C. T. Atkinson, N. J. Thomas, and D. B. Hunter (eds.). Blackwell Publishing, Ames, Iowa, p. 54–107.

França, C. 1912. *Leucocytozoon* du geai, de l'épervier et la bécasse. Bulletin de la Société de Pathologie Exotique **5:** 17–21.

Garnham, P. C. C. 1966. Malaria parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford, U.K., 1,114 p.

Godfrey, R. D., A. M. Fedynich, and D. B. Pence. 1987. Quantification of hematozoa in blood smears. Journal of Wildlife Diseases **23:** 558–565.

Greiner, E. C., and A. A. Kocan. 1977. *Leucocytozoon* (Haemosporida; Leucocytozoidae) of the Falconiformes. Canadian Journal of Zoology **55:** 761–770.

HELLGREN, O. 2005. The occurrence of haemosporidian parasites in the Fennoscandian bluethroat (*Luscinia svecica*) population. Journal of Ornithology **146:** 55–60.

———, A. KRIŽANAUSKIENĖ, G. VALKIŪNAS, AND S. BENSCH. 2007. Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida, Haemoproteidae). Journal of Parasitology **93:** 889–896.

ISHAK, H. D., J. P. DUMBACHER, N. L. ANDERSON, J. J. KEANE, G. VALKIŪNAS, S. HAIG, L. A. TELL, AND R. N. M. SEHGAL. 2008. Blood parasites in owls with conservation implications for the spotted owl (*Strix ocidentalis*). PloS ONE **3:** e2304.

KRONE, O. J., J. WALDENSTRÖM, G. VALKIŪNAS, O. LESSOW, K. MÜLLER, T. A. IEZHOVA, J. FICKEL, AND S. BENSCH. 2008. Haemosporidian blood parasites in European birds of prey and owls. Journal of Parasitology **94:** 709–715.

KUMAR, S., K. TAMURA, AND M. NEI. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5:** 150–163.

MARTINSEN, E. S., I. PAPERNA, AND J. J. SCHALL. 2006. Morphological versus molecular identification of avian Haemosporidia: An exploration of three species concepts. Parasitology **133:** 279–288.

PEIRCE, M. A., A. G. GREENWOOD, AND K. SWINNERTON. 1997. Pathogenicity of *Leucocytozoon marchouxi* in the pink pigeon (*Columba mayeri*) in Mauritius. Veterinary Record **140:** 155–156.

PERKINS, S. L. 2008. Molecular systematics of the three mitochondrial protein-coding genes of malaria parasites: Corroborative and new evidence for the origins of human malaria. Mitochondrial DNA **19:** 471–478.

———, I. N. SARKAR, AND R. CARTER. 2007. The phylogeny of rodent malaria parasites: Simultaneous analysis across three genomes. Infection, Genetics and Evolution **7:** 74–83.

RAIDAL, S. R., AND S. M. JAENSCH. 2000. Central nervous disease and blindness in nankeen kestrels (*Falco cenchroides*) due to a novel *Leucocytozoon*-like infection. Avian Pathology **29:** 51–56.

RICKLEFS, R. E., S. M. FALLON, AND E. BERMINGHAM. 2004. Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. Systematic Biology **53:** 111–119.

SEHGAL, R. N. M., A. C. HULL, N. L. ANDERSON, G. VALKIŪNAS, M. J. MARKOVETS, S. KAWAMURA, AND L. A. TELL. 2006. Evidence for cryptic speciation of *Leucocytozoon* spp. (Haemosporida, Leucocytozoidae) in diurnal raptors. Journal of Parasitology **92:** 375–379.

———, G. VALKIŪNAS, T. A. IEZHOVA, AND T. B. SMITH. 2006. Blood parasites of chickens in Uganda and Cameroon with molecular descriptions of *Leucocytozoon schoutedeni* and *Trypanosoma gallinarum*. Journal of Parasitology **92:** 1336–1343.

STEELE, E. J., AND G. P. NOBLET. 1993. Gametocytogenesis of *Leucocytozoon smithi*. Journal of Eukariotic Microbiology **40:** 384–391.

SWOFFORD, D. L. 2002. PAUP*. Phylogenetic analysis using parsimony (and other methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

TARELLO, W. 2006. *Leucocytozoon toddi* in falcons from Kuwait: Epidemiology, clinical signs and response to melarsomine. Parasite **13:** 179.

VALKIŪNAS, G. 2005. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton, Florida, 946 p.

———, C. T. ATKINSON, S. BENSCH, R. N. M. SEHGAL, AND R. E. RICKLEFS. 2008. Parasite misidentifications in GenBank: How to minimize their number? Trends in Parasitology **24:** 247–248.

———, T. A. IEZHOVA, A. KRIŽANAUSKIENĖ, V. PALINAUSKAS, R. N. M. SEHGAL, AND S. BENSCH. 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology **94:** 1395–1401.

———, ———, C. LOISEAU, AND R. N. M. SEHGAL. 2009. Nested cytochrome b polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. Journal of Parasitology **94:** 1512–1515.

———, ———, ———, T. B. SMITH, AND R. N. M. SEHGAL. 2009. New malaria parasites of the subgenus *Novyella* in African rainforest birds, with remarks on their high prevalence, classification and diagnostics. Parasitology Research **104:** 1061–1077.

———, A. KRIŽANAUSKIENĖ, T. A. IEZHOVA, O. HELLGREN, AND S. BENSCH. 2007. Molecular phylogenetic analysis of circumnuclear hemoproteids (Haemosporida: Haemoproteidae) of sylviid birds, with a description of *Haemoproteus parabelopolskyi* sp. nov. Journal of Parasitology **93:** 680–687.