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BLOOD PARASITES OF CHICKENS IN UGANDA AND CAMEROON WITH MOLECULAR DESCRIPTIONS OF *LEUCOCYTOZOON SCHOUTEDENI* AND *TRYPANOSOMA GALLINARUM*

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ABSTRACT: Using microscopy and PCR, we determined the prevalence of blood parasites in village chickens in Uganda and Cameroon. Of 148 individuals tested, 18.3% were infected with *Leucocytozoon schoutedeni* (Haemosporida, Leucocytozoidae) and 4.1% were infected with *Trypanosoma gallinarum* (Kinetoplastida, Trypanosomatidae). No other blood parasites were detected. Subsequent phylogenetic analysis of the cytochrome *b* gene of *L. schoutedeni* identified 2 distinct lineages that were found at all 3 sampling locations in Uganda. The sequence divergence between these 2 lineages is 1.5%. One of these lineages was also found in chickens in Cameroon, nearly 2,000 km distant. There are no morphological differences between blood stages of the parasites represented by the 2 different lineages, suggesting that cytochrome *b* gene sequence divergence can be as high as 1.5% within a single well-defined morphospecies of *Leucocytozoon*. We sequenced a portion of the small subunit ribosomal RNA gene (SSU rRNA) of *T. gallinarum*, and redescribe *T. gallinarum* for the first time since its discovery in 1911. These are the first assignments of DNA sequence data to these morphospecies of *Leucocytozoon* and *Trypanosoma* and may represent an example of intraspecific sequence divergence.

Many recent studies have focused on avian blood parasites as a model system for host–parasite interactions in an evolutionary and ecological context (i.e., Bensch et al., 2004; Hellgren et al., 2004; Ricklefs et al., 2005). Relative to the published information on wild birds, a much larger literature exists on these parasites in poultry. Extensive laboratory studies have been conducted describing their pathologies, especially for species of *Leucocytozoon* (Garnham, 1966; Noblet et al., 1976; Morii, 1992; Nakamura et al., 1997; Ito and Gotanda, 2005). Based on the current taxonomy, 3 species of *Leucocytozoon* and 3 species of *Trypanosoma* are found in the domestic chicken *Gallus gallus domesticus*, mainly in tropical and subtropical regions worldwide. These are *Leucocytozoon macleani* Sambon, 1908; *Leucocytozoon caulleryi* Mathis and Léger, 1909; *Leucocytozoon schoutedeni* Rodhain, Pons, Vandenbranden and Bequaert, 1913; *Trypanosoma numidae* Wenyon, 1908; *Trypanosoma calmettei* Mathis and Léger, 1909; and *Trypanosoma gallinarum* Bruce, Hamerton, Bateman, Mackie and Bruce, 1911. These species are well distinguished based on the morphology of their blood stages and/or laboratory experiments documenting their transmission and life cycles (Baker, 1976; Valkiūnas, 2005).

Although the pathogenicity of many species of the Leucocytozoidae (Sporozoa, Haemosporida) in wild birds is unclear, many cases of mortality have been reported in domestic chickens and other poultry (Garnham, 1966; Bennett et al., 1993; Valkiūnas, 2005). *Leucocytozoon caulleryi* is especially virulent; infected chickens frequently show severe signs of anorexia, ataxia, and anemia and have difficulty breathing. They frequently die because of hemorrhages as a result of rupture of megalomeronts that may develop in all organs and tissues (Garnham, 1966; Morii, 1992). In addition, birds may be susceptible to secondary infections that may increase mortality. *Leucocytozoon schoutedeni* and *L. macleani* are much less pathogenic (Valkiūnas, 2005). The majority of *Leucocytozoon* spp. are transmitted by blackflies (Simuliidae). Only *L. caulleryi* (a chicken-specific parasite) is spread by biting midges belonging to the Ceratopogonidae (Morii, 1992; Valkiūnas, 2005).

The most common vectors of avian trypanosomes are arthropods belonging to the Hippoboscidae, Culicidae, Ceratopogonidae, and Simuliidae (Olsen, 1974; Baker, 1976). In addition, dermanyssid mites have been identified as avian trypanosome vectors (Molyneux, 1977). Little is known concerning the pathogenic effects of trypanosomes in chickens, although artificial infection with *Trypanosoma brucei* showed no obvious impairment of health (Joshua et al., 1982). Because trypanosomes are generally rare in peripheral blood, but are typically much more abundant in bone marrow; detection methods relying solely on blood may underestimate the true prevalence of these parasites (Baker, 1976; Apanius, 1991).

Previous accounts of blood parasites in chickens in Africa are relatively scarce. In a study in Zimbabwe, 4 of 94 chickens harbored *Leucocytozoon sabrazesi* Mathis and Léger, 1910 (= *L. macleani*) and 5 of the 94, *Trypanosoma* sp. (Permin et al., 2002). But in Ghana, no *Leucocytozoon* or *Trypanosoma* infections were detected (Poulsen et al., 2000). Earlier studies showed *Leucocytozoon* spp. infected 55 of 163 (34%) examined chickens in Ibadan, Nigeria (Adene and Dipeolu, 1975), but of 110 chickens observed in Anambra, Nigeria, none was infected (Orajaka and Nweze, 1991). A study in Mozambique reported 1 chicken infected with what may have been *Leucocytozoon andrewsi* Atchley, 1951 (= *L. schoutedeni*) (Son, 1960). Fallis et al. (1973) reported that of 150 chickens tested in Tanzania, more than 50% were infected with *L. schoutedeni*.

Recently, DNA sequencing has provided more information regarding the evolution and phylogenetic relationships of avian blood parasites. As with avian Haemoproteidae species, the concept of host family/host subfamily specificity for *Leucocytozoon* spp. was widely accepted in taxonomic studies in the 20th century (Desser and Bennett, 1993), but has been questioned as a device for taxonomy of hemosporidian parasites by current studies (Valkiūnas and Ashford, 2002; Szymanski and Lovette, 2005). In addition, it has been suggested that many more species or subspecies of blood parasites may exist than originally described, with many instances of cryptic speciation

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TABLE I. Occurrence of blood parasites in chickens, *Gallus gallus domesticus*, in Uganda (July 2003) and Cameroon (July 2005).

Site (village bordering study site)	No. examined	No. infected	Parasite present	
			Leucocytozoon schoutedeni	*Trypanosoma allinarum*
Uganda				
Kibale National Park	31	13 (41.9)*	11 (35.5)	5 (16.1)
Bwindi Impenetrable National Park	28	4 (14.3)	4 (14.3)	0
Mabira Forest	18	8 (44.4)	7 (38.9)	1 (5.6)
Total	77	25 (32.5)	22 (28.6)	6 (7.8)
Cameroon				
Nkouak	40	2 (5.0)	2 (5.0)	0
Zoebefame	31	3 (9.7)	3 (9.7)	0
Total	71	5 (7.0)	5 (7.0)	0
Grand total	148	30 (20.3)	27 (18.3)	6 (4.1)

* Percentage of birds positive.

(Perkins, 2000; Bensch et al., 2004; Ricklefs et al., 2005; Sehgal et al., 2006). Based on studies using the mitochondrial cytochrome *b* gene, the leucocytozooids are basal to *Plasmodium* and *Haemoproteus* spp. (Perkins and Schall, 2002) and studies in Sweden and California have described many lineages of *Leucocytozoon* spp. (Hellgren et al., 2004; Sehgal et al., 2006). In these investigations, however, questions arise about what constitutes a distinct species of *Leucocytozoon*. Recently, helping to elucidate these questions, a 0.89% sequence divergence was observed among 4 cytochrome *b* lineages of the morphospecies *Leucocytozoon gentili* from house sparrows (*Passer domesticus*) in Israel (Martinsen et al., 2006). Although over 30 species of *Leucocytozoon* have been described based on the morphology of their blood stages and limited experimental information about their vertebrate-host specificity, degrees of intraspecific and interspecific diversity within the same species of *Leucocytozoon* and other hematozoa remain unclear. This information is important to understand the speciation of blood hemosporidian parasites and the genetic bounds for their morphospecies. Because *L. schoutedeni* is a readily distinguishable morphospecies, it provides a good model for the investigation of intraspecific genetic diversity of leucocytozooids.

Several recent studies have focused on the phylogenetics of *Trypanosoma* spp. in birds (Sehgal et al., 2001; Votypka et al., 2002, 2004; Hamilton et al., 2005). These studies have used the 18S rRNA gene and to some extent kinetoplast DNA to infer relationships among the *Trypanosoma* spp. Here, we study the prevalence and morphology of 2 distinct morphospecies of the chicken blood parasites *L. schoutedeni* and *T. gallinarum*, assign DNA sequence information to them, and discuss sequence divergence and phylogenetic relationships in these parasites.

MATERIALS AND METHODS

Collection of blood samples and extraction of DNA

The blood samples used in this study were collected at 3 sites in Uganda in July 2003 (see Valkiūnas et al., 2005) and 2 sites in Cameroon in July 2005 (Table I). In Uganda, blood was taken from chickens in villages immediately adjacent to Bwindi Impenetrable National Park (1°3.5′S, 29°46.5′E; 2,340 m above sea level), Kibale National Park (0°34.7′N, 30°21.3′E; 1,580 m), and Mabira Forest Reserve (0°30′N, 33°0′E; 1,070 m). In Cameroon, the villages (again adjacent to contiguous rainforests) were Nkouak (3°52′N, 13°18′58″E) and Zoebefame (2°39′N, 13°23′49″E).

The blood samples were collected from active birds that were provided by their owners. Blood samples from all birds (50–100 μl) were collected by venipuncture of the brachial vein and stored in lysis buffer (10 mM Tris-Hcl, pH 8.0, 100 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulphate) for subsequent molecular analysis (Sehgal et al., 2001). To obtain total DNA, the blood was extracted following a DNeasy kit protocol (Qiagen, Valencia, California).

Three blood films were prepared from each bird. Blood films were air-dried within 5–10 sec after preparation. In humid environments, we used a battery-operated fan to aid in the drying of the blood smears. Slides were fixed in methanol in the field and then stained with Giemsa in the laboratory. Blood films were examined for 10–15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1,000). 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, as recommended by Godfrey et al. (1987). Species of *Trypanosoma* and *Leucocytozoon* were identified according to Baker (1976) and Valkiūnas (2005), respectively.

Parasite morphology and statistical analysis

An Olympus BX51 light microscope equipped with digital camera, and the amplification software DP-SOFT was used to prepare illustrations and to take measurements. The morphometric features studied (Tables II, III) were those defined by Baker (1976) and Valkiūnas (2005). Prevalences were compared by Yates' corrected chi-square test. 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. Representative blood slides of *L. schoutedeni* (accession numbers 6667, 6668, 7272, 7274, 7444, 7760–7762, 7445, NS) were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania.

PCR and sequencing

Extracted DNA was used in nested PCR reactions to amplify DNA fragments according to methods described by Hellgren et al. (2004) for *Leucocytozoon* spp. and by Sehgal et al. (2001) for *Trypanosoma* spp. PCR products were purified using a Qiagen kit following manufacturer's instructions. Bidirectional sequencing of the PCR fragments was performed using the primers HaemFL and HaemR2L for *Leucocytozoon* spp. (Hellgren et al., 2004) and S-755 and S-823 for *Trypanosoma* spp. (Sehgal et al., 2001) in an ABI Prism 377 automated sequencer (Applied Biosystems, Inc., Foster City, California). For PCR products from *Trypanosoma* spp., initial attempts to directly sequence PCR products yielded illegible sequences. In order to obtain clean sequences, we subcloned the fragments using a TOPO® TA-cloning kit (Invitrogen, Carlsbad, California) prior to sequencing. For each sample, at least 8 colonies

TABLE II. Morphometry of trypomastigotes (n = 8) of *Trypanosoma gallinarum* from chickens in Uganda.

Feature*	Measurement (μm)†
AK	0.6–1.1 (0.8 ± 0.2)
AN	15.3–26.6 (20.1 ± 4.0)
AT	110.9–247.8 (170.9 ± 41.7)
BW	3.8–7.8 (5.8 ± 1.3)
FF	7.8–12.2 (10.1 ± 1.7)
KN	14.0–21.9 (17.9 ± 2.2)
NA	30.6–40.7 (34.7 ± 3.7)
PA	54.5–76.3 (64.7 ± 7.9)
PK	10.1–17.8 (12.6 ± 3.0)
PN	24.2–36.5 (29.6 ± 4.2)
AN/AT‡	0.1–0.14 (0.1 ± 0.01)
BW/PA	0.07–0.1 (0.1 ± 0.01)
PK/PA	0.2–0.24 (0.2 ± 0.02)
PN/KN	1.5–1.9 (1.6 ± 0.2)
PN/NA	0.7–1.0 (0.9 ± 0.1)
PN/PA	0.4–0.5 (0.5 ± 0.03)

* Features: AK = area of kinetoplast; AN = area of nucleus; AT = area of trypomastigote; BW = width of body through center of nucleus; FF = free flagellum; KN = kinetoplast to center of nucleus; NA = center of nucleus to anterior end; PA = total length without free flagellum; PK = posterior end to kinetoplast; PN = posterior end to center of nucleus; BW/PA = body width index; PK/PA, PN/NA, PN/PA = nuclear index; PN/KN = kinetoplast index.

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

‡ The indices are calculated as a ratio of the first feature to the second feature.

were tested by PCR; transformed cells were used to seed the reactions. We used double-stranded cycle sequencing with dye–terminator fluorescent labeling, and electrophoresed sequenced products through a 5% PAGE Plus gel (Amresco, Solon, Ohio). Sequences are deposited in GenBank with the following accession numbers: DQ676823 and DQ676824 for the 2 lineages of *L. schoutedeni*, DQ676825 for *L. macleani*, and DQ676826–DQ676829 for the 4 lineages of *T. gallinarum*.

Phylogenetic analysis

For *Trypanosoma* spp., because samples were collected in remote field locations, culturing of the parasites was impractical. Therefore, because of low parasitemias in the samples, we obtained relatively short fragments (370 bp) of the SSU rRNA gene by PCR (Sehgal et al., 2001). Sequences were aligned, and because only a small number of nucleotide substitutions separated the SSU rRNA lineages, we did not obtain well-supported hierarchical structure in tree-based phylogenetic reconstructions for different lineages of *T. gallinarum*. Pairwise base differences among lineages were determined using PAUP*4.0b10 (Swofford, 2002). For comparison of trypanosome sequences, the following accession numbers were used: AF416559 and AF361423 for *Trypanosoma avium*, and AJ223562 for *Trypanosoma bennetti*.

We based our phylogenetic analysis of *Leucocytozoon* spp. on sequences (472 nucleotides) of the cytochrome *b* gene from 24 individuals, plus 1 sequence from the scaly francolin *Francolinus squamatus*. The sequences with the following accession numbers were also used in the analyses: AY393796 for *Leucocytozoon* sp. lineage 1SISKIN2, AY733088 for *Plasmodium relictum*, DQ451436 for *Leucocytozoon gentili*, AY099063 for *Leucocytozoon dubreuli*, DQ451439 for *Leucocytozoon majoris*, DQ451432 for *L. squamatus*, and DQ355976 for a *Leucocytozoon* found in Attwater's prairie-chicken. Sequences were aligned using the program SEQUENCHER version 3.0 (Gene Codes Corporation, Ann Arbor, Michigan). Phylogenetic analyses using maximum-parsimony techniques were conducted using PAUP*4.0b10 (Swofford, 2002). Searches used the bootstrap search option with 500 stepwise addition replicates using the TBR branch-swapping algorithm. Simple consensus trees were constructed to summarize the results. In addition, we performed distance analyses using the Kimura 2-parameter distance model, and taxa were joined using neighbor-joining. We also did a maximum likelihood analysis with a heuristic search of 100 ran-

TABLE III. Morphometry of gametocytes and host cells of 2 lineages of *Leucocytozoon schoutedeni* from chickens in Uganda.

Feature	Measurement (μm)*			
	Lineage 1		Lineage 2	
	Macrogametocyte (n = 31)	Microgametocyte (n = 31)	Macrogametocyte (n = 31)	Microgametocyte (n = 25)
Parasite				
Length	11.9–15.3 (13.1 ± 0.8)	9.9–13.4 (11.8 ± 0.8)	11.8–14.8 (13.3 ± 0.7)	10.1–15.4 (12.2 ± 1.2)
Width	9.8–13.0 (11.8 ± 0.6)	8.0–11.1 (10.0 ± 0.7)	9.1–13.2 (11.8 ± 0.9)	7.7–11.4 (10.1 ± 0.8)
Perimeter	40.6–47.2 (43.5 ± 1.4)	34.4–41.6 (38.2 ± 1.8)	38.9–46.7 (43.5 ± 2.2)	32.7–42.9 (39.0 ± 2.3)
Area	104.2–133.7 (120.8 ± 7.0)	72.6–110.7 (95.0 ± 9.6)	98.8–138.9 (122.9 ± 11.1)	72.5–128.7 (99.0 ± 12.3)
Parasite nucleus				
Length	2.8–6.5 (4.0 ± 0.8)	6.4–10.2 (8.0 ± 1.0)	2.7–5.9 (4.0 ± 0.7)	5.1–12.2 (8.7 ± 1.5)
Width	2.0–3.3 (2.7 ± 0.4)	4.0–7.7 (6.0 ± 0.8)	1.9–4.1 (2.9 ± 0.5)	4.2–7.5 (6.1 ± 0.9)
Area	7.6–16.4 (10.4 ± 2.1)	32.5–59.3 (42.0 ± 7.7)	5.5–18.1 (10.0 ± 2.9)	17.2–82.7 (45.9 ± 13.5)
Host-parasite complex				
Area	150.1–254.5 (208.7 ± 22.8)	136.0–224.6 (184.3 ± 20.8)	167.3–276.2 (213.3 ± 24.4)	113.3–225.2 (180.1 ± 28.9)
Host cell nucleus				
Length	17.7–25.0 (21.1 ± 1.9)	16.5–27.5 (21.4 ± 2.2)	14.6–25.8 (20.5 ± 2.7)	15.1–26.4 (20.5 ± 2.7)
Width	1.5–4.2 (2.6 ± 0.7)	1.5–3.9 (2.7 ± 0.6)	1.4–3.7 (2.5 ± 0.5)	1.0–3.5 (2.6 ± 0.6)
Perimeter of parasite covered	16.2–23.3 (18.8 ± 1.7)	14.5–23.1 (18.0 ± 2.2)	13.4–22.8 (18.1 ± 2.2)	11.3–22.0 (17.3 ± 2.6)

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

dom stepwise addition replicates. These methods produced trees with identical topologies to the maximum parsimony results. Genetic divergences between lineages were estimated using the distance setting HYK85, which yielded similar results as the uncorrected ''p'' setting.

RESULTS

Prevalence and morphology of *Leucocytozoon* and *Trypanosoma* spp. in African village chickens

Seventy-seven chickens were examined from Uganda and 71 from Cameroon, of which 22 chickens were infected with *Leucocytozoon* and 6 with *Trypanosoma* in Uganda, and 5 with *Leucocytozoon* in Cameroon. PCR-based methods supported the results of microscopy, which are listed in Table I. Examination of blood films revealed that the chickens studied harbored *L. schoutedeni* and *T. gallinarum*. Figures 1–8 show microphotographs of *L. schoutedeni* and *T. gallinarum* found in chickens. Two samples also harbored mixed infections of *T. gallinarum* and a *Trypanosoma* sp. that clearly differed from the former by its size and pale staining of the cytoplasm. This trypanosome was not identified to species because only 1 trypomastigote of the parasite was seen in each sample. No other blood parasites were detected. There was no significant difference in prevalence of *L. schoutedeni* and *T. gallinarum* among different study sites in either Uganda or Cameroon. Both *L. schoutedeni* and *T. gallinarum* infections were significantly more prevalent in Uganda than in Cameroon ($P < 0.01$ and $P < 0.05$, respectively).

Table II details the morphometry of the trypomastigotes of *T. gallinarum*, which is recorded here for the first time in African chickens since its discovery. Below, we redescribe this parasite. Morphometry of gametocytes and their host cells of *L. schoutedeni* are given in Table III.

REDESCRIPTION

Trypanosoma gallinarum
Bruce, Hamerton, Bateman, Mackie and Bruce, 1911
(Figs. 7, 8; Table II)

Trypomastigotes easily found in thin blood films; few seen in infected birds, with 1–2 parasites recorded in each blood film. Only 8 nondeformed organisms seen and measured (Table II). Parasites large, spindle-shaped organisms with both posterior and anterior ends markedly drawn out and pointed. Cytoplasm stains dark blue with clear areas located around prominent kinetoplast; latter structure oval in outline, stains densely purple, and situated far from posterior end of cell (Figs. 7, 8). Centrally placed nucleus compact, usually roundish or oval. Longitudinal striations present on cell surface. Free flagellum originates just anterior to kinetoplast. Undulating membrane conspicuous, with well-pronounced undulations; undulation number varies (Figs. 7, 8), but always high (on average 8.9 ± 1.5). No dividing forms seen. Parasite multiplies in tsetse fly, *Glossina palpalis*, but role of this fly in natural transmission of parasite remains unknown (Duke, 1912). No information regarding pathogenicity in chickens.

Taxonomic summary

Type host: Gallus gallus domesticus L. (Galliformes, Phasianidae).

Type locality: Uganda; villages immediately adjacent to Kibale National Park and Mabira Forest.

Distribution: This parasite has been found only in Uganda so far.

Site of infection: Blood plasma.

Prevalence: Low both by microscopic examination and DNA amplification. The parasites are rare in the peripheral circulation.

Type specimens: Neohapantotype (accession number USNPC 096166.00, *G. g. domesticus*, 15.07.2003, Uganda, Kibale, coll. G. Valkiūnas) was deposited in the U.S. National Parasite Collection, Beltsville, Maryland. Paraneohapantotypes (G464756, *G. g. domesticus*, 15.07.2003, Uganda, Kibale, coll. G. Valkiūnas, and 7753 NS, *G. g. domesticus*, 3.08.2003, Uganda, Mabira Forest, coll. G. Valkiūnas) were deposited in the International Reference Centre for Avian Haematozoa, Queensland Museum, Queensland, Australia and in the Institute of Ecology, Vilnius University, Vilnius, Lithuania, respectively.

Remarks

The main differentiating characters of *T. gallinarum* are the large size of markedly spindle-shaped trypomastigotes (>60 μm in average; the largest organisms exceed 75 μm in length) and the numerous well-pronounced undulations of the undulating membrane (Figs. 7, 8; Table II). These characters were illustrated and reported in the original description (Bruce et al., 1911). Based on these features, *T. gallinarum* can be distinguished from all morphospecies of trypanosomes tentatively belonging to the *T. avium* group, which unites large parasites with a kinetoplast located far from the hind end of the body (Baker, 1976). *Trypanosoma gallinarum* can be readily distinguished from other morphospecies of trypanosomes described from chickens, *T. calmettei* and *T. numidae*, by its large size, the position of the kinetoplast, and the architectonics of undulations of the undulating membrane. The total length without free flagellum of trypomastigotes of *T. calmettei* is <30 μm, and the kinetoplast is situated close to the posterior end of the body. The total length without free flagellum of trypomastigotes of *T. numidae* is <54 μm, and fewer undulations of the undulating membrane (≤5) are present (Wenyon, 1908). These features are not characteristic of *T. gallinarum*.

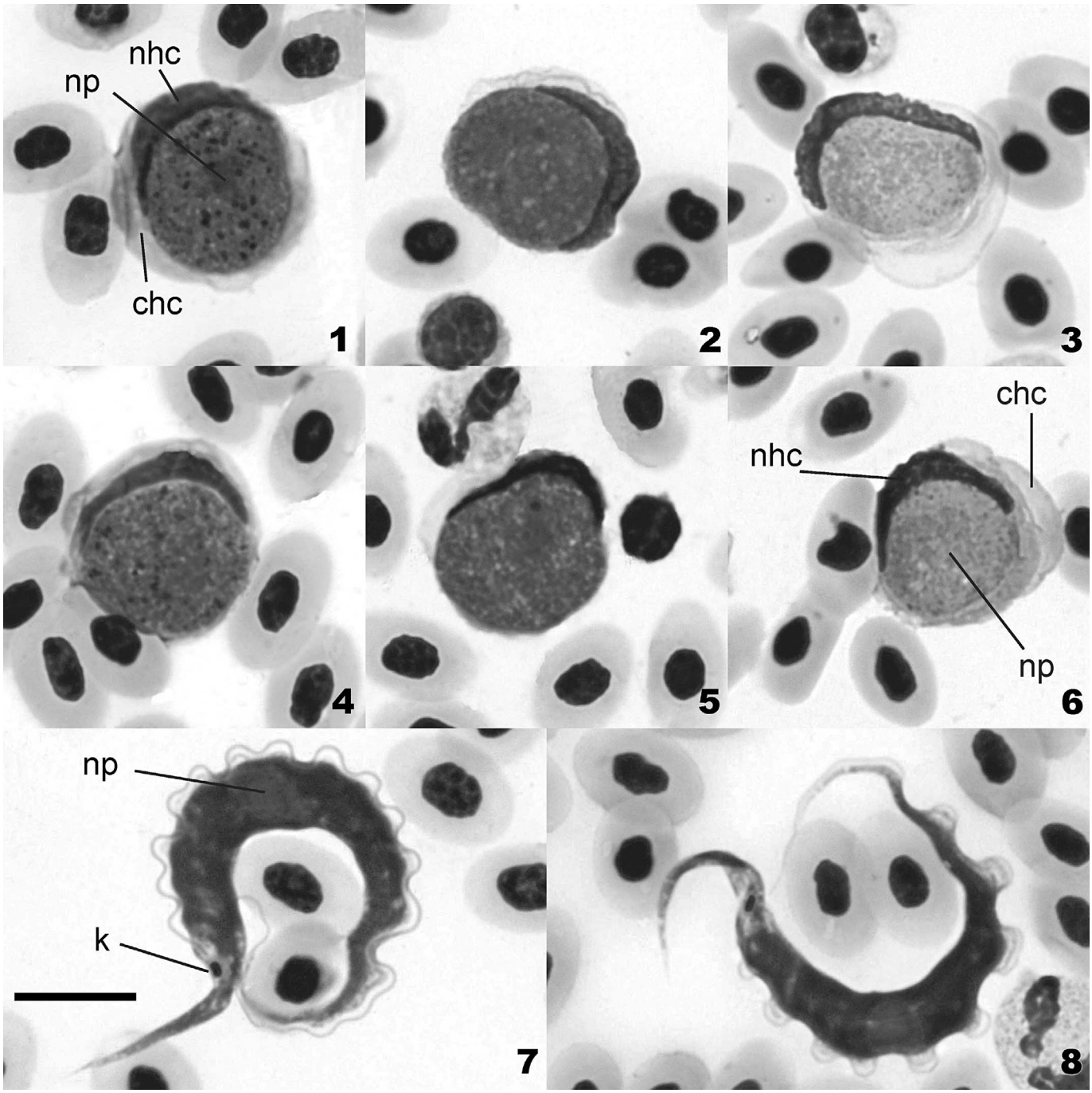
We were able to obtain sequences from 5 of the 6 chickens infected with *T. gallinarum* in Uganda (Kibale, Table I). Of these, we obtained 4 almost identical sequences. Lineage 1 was shared by 2 infected chickens. Lineage 2 differed from Lineage 1 by 1 base change. Lineage 3 differed from Lineage 1 by 2 base changes, and Lineage 4 differed by 5 base changes. Lineage 1 differed from our previously published SSU rRNA sequence of *T. avium* from the olive sunbird, *Nectarinia olivacea* (GenBank AF361423) found in Equatorial Guinea, by 9 base changes, a 3.2% sequence divergence. They differed from another lineage of *T. avium* (AF416559) (obtained from a lesser spotted eagle, *Aquila pomarina*, from Slovakia) by an average of 22.5 base pair changes, a 5.4% sequence divergence. Sequence divergence between *T. gallinarum* and 2 other avian trypanosomes, *T. bennetti* (AJ223562) and *T. everetti* (AF361430), was 8.5% and 9.1% respectively. The specimens corresponding to Lineages 1 and 4 contained mixed infections with the other unidentified *Trypanosoma* spp. as determined by microscopy. The specimens containing *Trypanosoma* sp. of Lineages 2 and 3 were void of mixed infections both by microscopy and PCR, so we conclude that these 2 lineages are representative of *T. gallinarum*. High sequence similarity suggests that Lineages 1 and 4 are also *T. gallinarum*, but we can not rule out that these may be sequences of the scarce parasite visible in these samples.

We sequenced PCR products of the cytochrome *b* gene of *L. schoutedeni* from 24 infected individuals and identified 2 distinct lineages. No double peaks were observed in the chromatograms of these sequences. Both of the 2 lineages were present at all 3 sampling locations in Uganda, some within a single chicken coop. Lineage 1 was found in 18 chickens and Lineage 2 was found in 6. In Cameroon, we obtained sequence data from 4 infected chickens, all parasites with Lineage 1. Phylogenetic analysis showed strong support for the 2 distinct lineages of parasites (Fig. 9). The sequence divergence between the 2 lineages was 1.5%. The average sequence divergence between *L. schoutedeni* from chickens and *L. macleani* from a scaly francolin in Cameroon was 7.4% and between the *L. schoutedeni* lineages and Lineage 1SISKIN2 of a passerine *Leucocytozoon* species was 5.8%. The average sequence divergence between a parasite found in Attwater's prairie-chicken and *L. schoutedeni* was 6.9%. Between published sequences of 2 distinct morphospecies, *L. dubreuli* and *L. gentili* we measured a sequence divergence of 3.3%. Morphometric analysis of the parasites revealed no detectable morphological differences in all features studied between the parasites of the 2 *L. schoutedeni* lineages (Table III).

DISCUSSION

Prevalence and geographical distribution of parasites

The chicken samples examined in this study lacked *Haemoproteus* and *Plasmodium* spp. infections. In a study in Zim-



FIGURES 1–8. Photomicrographs of gametocytes of *Leucocytozoon schoutedeni* (**1–6**) and trypomastigotes of *Trypanosoma gallinarum* (**7, 8**) from the blood of chickens. **1–3.** Lineage 1. **4–6.** Lineage 2. **1, 2, 4, 5.** Macrogametocytes. **3, 6.** Microgametocytes. Chc = cytoplasm of host cell, k = kinetoplast, nhc = nucleus of host cell, np = nucleus of parasite. Methanol-fixed and Giemsa-stained thin blood films. Bar = 10 μm.

babwe, 14 of 94 chickens harbored *Plasmodium gallinaceum* Brumpt, 1935 (Permin et al., 2002), and in Ghana 27 of 100 birds harbored *Plasmodium juxtanucleare* Versiani and Gomes, 1941 (Poulsen et al., 2000). The chickens of this study were collected from several habitats where mosquitoes and biting midges were abundant (data not shown), so the lack of *Plasmodium* and *Haemoproteus* spp. infections of these birds from Uganda and Cameroon is unexpected. In general, the blood parasites of African chickens are not well studied, considering the availability of materials. Our results and the literature (Son, 1960; Fallis et al., 1973; Adene and Dipeolu, 1975; Huchzermeyer, 1993; Permin et al., 2002) suggest that *Leucocytozoon* spp. may be the most common hematozoan in these birds in Africa. The higher prevalence of blood parasites in chickens in Uganda is consistent with our earlier studies, which have shown that the prevalence of avian blood parasites in Uganda (61.9%) (Valkiūnas et al., 2005) is higher than in Cameroon (28.6% [Sehgal et al., 2005] and 17% [Kirkpatrick and Smith, 1988]).

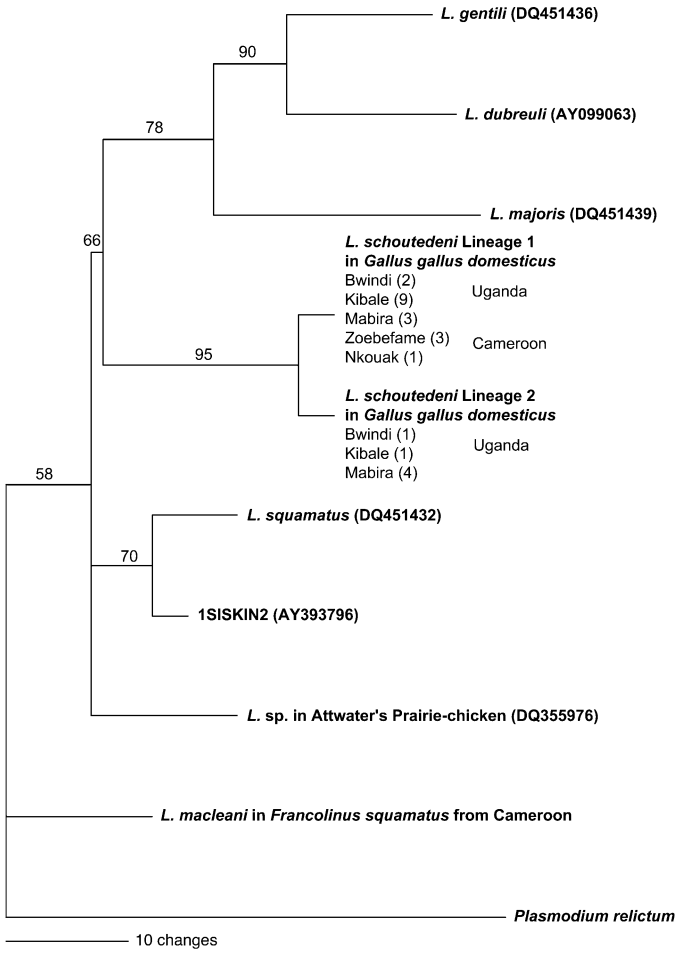


FIGURE 9. Consensus maximum parsimony bootstrap tree based on sequences (472 bp) of *Leucocytozoon schoutedeni* cytochrome *b* gene sequences from 24 chickens. The tree was rooted by *Plasmodium relictum* from a captive African penguin *Spheniscus demersus*. Also included are cytochrome *b* sequences of several other *Leucocytozoon* species. Bootstrap percentages of clades (500 iterations) are shown above internal nodes. Of the 24 novel sequences, there are 2 distinct lineages. Both lineages were found at all 3 sampling locations in Uganda, and Lineage 1 was also isolated from 3 chickens from Cameroon (numbers in parentheses represent the number of individuals from each location). Analyses using maximum likelihood and neighbor-joining yielded identical topologies (data not shown).

It is probable that more active transmission of blood parasites takes place in Uganda, which may be because of different vector ecologies in these countries and warrants further investigation. We did not find any chicken parasites in forest bird species (data not shown), and the francolin (*Francolinus squamatus*) harbored a morphologically distinct *Leucocytozoon*, *L. macleani*.

It is worth noting that species of *Leucocytozoon* in chickens are widespread in the tropical and subtropical regions of the Old World, but are scarce in the central and northern Palearctic and the Americas (Valkiūnas, 2005). It is possible that the chicken leucocytozoids evolved with their insect vectors in warm climates in the Old World, are not able to develop in the temperate climate of the Palearctic, and have not yet penetrated the New World. However, the parasites have been introduced recently to new areas, as observed with *L. schoutedeni* in South Carolina (Atchley, 1951; Noblet et al., 1976).

Avian trypanosomes are widespread worldwide (Baker, 1976), but they have only been reported sporadically in chickens in the tropics of the Old World. Interestingly, a single report of *T. calmettei* and a few reports of *T. gallinarum* are known only from Vietnam and Uganda, respectively (Mathis and Léger, 1909; Bruce et al., 1911; Duke, 1912). Unidentified trypanosomes were reported in chickens from Borneo, Indonesia (Bennett et al., 1982). Permin et al. (2002) reported trypanosomes identified as *Trypanosoma avium* Danilewsky, 1885 in chickens in Zimbabwe, but provided no evidence for the species identification. It is possible that these authors were in fact studying *T. gallinarum*. A few records of trypomastigotes, which were attributed to *T. gallinarum*, in Holarctic tetraonid birds are most likely misidentifications because these parasites either were not described (Oliger, 1940, 1957) or are of smaller size (Clarke, 1935) than the organisms that were originally described and illustrated in Ugandan chickens (Bruce et al., 1911). Given the cosmopolitan distribution of avian trypanosomes, the scarcity of these parasites in chickens in the tropics of the Old World and the lack of these parasites in chickens in the Holarctic and New World is compelling. Thus, new records and observations of chicken *Trypanosoma* spp. are of particular theoretical interest. The type material for all chicken trypanosomes, except *T. gallinarum*, is lacking.

Morphology of blood stages of parasites

Trypanosoma gallinarum was discovered in the domestic chicken in Uganda by Bruce et al. (1911) and then briefly studied by Duke (1912). Since then, this parasite has not been recorded in chickens. The type material of this morphospecies is designated for the first time in this study. *Trypanosoma gallinarum* can be readily distinguished from other trypanosomes of chickens, primarily by the huge size of its trypomastigotes (Figs. 7, 8; Table II; Baker, 1976).

Leucocytozoon schoutedeni was well characterized by Fallis et al. (1973), who studied its development in both domestic chickens and the insect vector. Parasites of the 2 lineages observed here were morphologically indistinguishable from each other and from those described from chickens by Rodhain et al. (1913), Fallis et al. (1973), and Valkiūnas (2005). Based on the morphology of its blood stages, the recorded parasite certainly belongs to *L. schoutedeni*, which was first described by Rodhain et al. (1913) in the former Belgian Congo, and appears to be the most common *Leucocytozoon* morphospecies in African chickens (Fallis et al., 1973; Huchzermeyer, 1993; Valkiūnas, 2005). This is the first study quantitatively documenting the morphological similarity of blood stages of different lineages of the same *Leucocytozoon* morphospecies (Table III; Figs. 1–6).

Lineage diversity of parasites

We observed sequence variation in the SSU rRNA gene among the 5 *T. gallinarum* samples tested here. Two of the 4 lineages presented here are of samples that harbored mixed infections with unidentified species of *Trypanosoma*. Because of the low intensity of infection, we did not obtain the sequence of the second *Trypanosoma* sp. despite efforts subcloning PCR

fragments. Based on their close morphological and sequence similarity to the 2 other lineages taken from samples with single infections, we conclude that all 4 lineages are of *T. gallinarum*. The small differences among the 4 lineages may represent differences in the 2 loci of the SSU rRNA gene. Our previous work showed little host or geographic specificity of *Trypanosoma* species (Sehgal et al., 2001). Because of the extensive movement of chickens with people, we cannot speculate about the phylogeography of their parasites. Although the obtained sequences are not conducive to extensive phylogenetic analyses, among the published SSU rRNA sequences, *T. gallinarum* most closely resembles *T. avium*. Sequence divergence between *T. gallinarum* and an African lineage of *T. avium* was 3.2%, and for a European lineage of *T. avium*, 5.4%. Clearly, the phylogenetic relationships among avian trypanosomes are largely unresolved and additionally hampered mainly by the presence of simultaneous infections, light parasitemias, and the pleiomorphic nature of the parasites.

Of particular interest is the finding of 2 distinct lineages of *L. schoutedeni* in Uganda, and 1 of these same lineages in Cameroon (Fig. 9). A possible ecological explanation for this is that the 2 lineages of this morphospecies evolved allopatrically in different regions of Africa, but came recently into sympatry as a result of human-mediated movements of poultry (Martens and Hall, 2000); this warrants further investigation. Another possibility is that the 2 lineages of *L. schoutedeni* in Uganda represent 2 cryptic species. For instance, Bensch et al. (2004) reported that within the morphologically well-defined species, *Haemoproteus payevskyi* Valkiūnas, Iezhova and Chernetsov, 1994, lineages of cytochrome *b* exhibiting less than 0.5% sequence divergence were associated with different sequences at the nuclear dihydrofolate reductase–thymidylate synthase (DHFR-TS) locus. Because these lineages do not recombine, the authors suggest that they are evolutionarily distinct species. This may also be the case with *L. schoutedeni*.

What constitutes a distinct protozoan species is contentious and ideally would be based on combining molecular data with information on basic life history strategies of these organisms as well as on morphology and results of controlled infection experiments. Genetic bounds even for well-defined morphospecies of avian blood parasites remain unclear. We have not sequenced nuclear genes from *L. schoutedeni*, but based on the available experimental and morphological data (Fallis et al., 1973; Valkiūnas, 2005; see Table III) we assert that at least within this particular morphospecies, a 1.5% sequence divergence in cytochrome *b* is likely because of intraspecific variation. It is worth noting that a sequence divergence of 8.4% was found between 2 lineages of 2 putative species of *Leucocytozoon* found in bluethroats, *Luscinia svecica* (Hellgren et al., 2004). Here we measured a divergence of 3.3% between the 2 morphospecies *L. dubreuli* and *L. gentili*, both parasites of passeriform birds (Valkiūnas, 2005). A higher cytochrome *b* sequence divergence of 10.9% was seen in the morphologically described *Leucocytozoon toddi*, an apparent example of cryptic speciation (Sehgal et al., 2006). In this study, the sequence divergence between 2 morphologically defined species, *L. macleani* and *L. schoutedeni*, was 7.4%.

Intraspecific genetic variation is a basic feature of natural populations, and is well described in the blood parasites of mammals (Joy et al., 2003). Here, we provide evidence for intraspecific variation in avian blood parasites. However, further studies linking DNA sequences with other morphologically and ecologically well defined species are needed to clarify what constitutes a distinct species based on cytochrome *b* sequence information. Because different species of birds belonging to the same genus frequently harbor different closely related lineages of *Haemoproteus* and *Leucocytozoon* spp. (Bensch et al., 2004; Ricklefs et al., 2005; Sehgal et al., 2006), the next substantive step will be to determine if and how host-dependent adaptations influence the lineage diversity of parasites, and how rapidly new lineages are established.

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