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Trypanosoma naviformis sp. nov. (Kinetoplastidae: Trypanosomatidae) from widespread African songbirds, the Olive sunbird (*Cyanomitra olivacea*) and Yellow-whiskered greenbul (*Andropadus latirostris*)

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Abstract

Trypanosoma naviformis **n. sp.** is described from the African olive sunbird *Cyanomitra olivacea* in Ghana based on the morphology of its hematozoic trypomastigotes and partial sequences of the small subunit ribosomal RNA gene. This parasite belongs to the group of small non-striated avian trypanosomes (< 30 μm in length in average) with the kinetoplast situated close to the posterior end of the body. *Trypanosoma naviformis* can be distinguished from other small avian trypanosomes due to its poorly visible flagellum, central position of its nucleus, and the symmetrically (in relation to the nucleus) narrowing of both ends of the hematozoic trypomastigotes, which are boat-like in shape. Illustrations of trypomastigotes of the new species are given, and SSU rDNA lineages associated with this parasite are documented. This parasite has been reported in Ghana and Cameroon and was also found in the yellow-whiskered greenbul, *Andropadus latirostris* in these countries. It appears to be widespread in its range given the distribution of these bird species in Africa.

Key words: *Trypanosoma*, bar coding, African birds, *Cyanomitra olivacea*, *Andropadus latirostris*

Introduction

Avian *Trypanosoma* parasites are cosmopolitan and some species have been reported to cause morbidity in birds (Baker, 1976; Molyneux *et al.*, 1983). Experimental studies (Fallis, *et al.*, 1973; Molyneux and Gordon, 1975) indicate that many morphotypes of bird trypanosomes can be readily distinguished in peripheral blood using morphological and morphometric characters, even during co-infections. These experimental studies indicate that the main morphological features of certain species remain constant during their development in different experimentally infected avian hosts. For example, hematozoic trypomastigotes of *Trypanosoma avium* never assume morphologies typical of *Trypanosoma everetti* or *Trypanosoma bouffardi* during single and simultaneous experimental infections, and they remain distinguishable. These experiments show that, despite some morphometric variations during development of the same *Trypanosoma* strains in different avian hosts, and marked variation of their morphology in vectors and in vitro cultures (Molyneux, 1977; Zidková *et al.*, 2012), the gross-morphological features of hematozoic trypomastigotes in the bloodstream are relatively stable. Thus, they can be used to identify new readily distinguishable morphotypes, as well as new parasite species or groups of species (Valkiūnas *et al.*, 2011). Identification of such readily distinguishable complexes of species is an important step in furthering the taxonomy of avian trypanosomes. *Trypanosoma everetti* is a well-known example of a species that was described solely based on morphological and morphometric characters (Molyneux, 1973).

During studies on the effects of deforestation on the prevalence of blood pathogens in African rainforest birds, large numbers of blood samples were collected from over 200 species of birds (Bonneaud *et al.*, 2009; Chasar *et al.*, 2009; Loiseau *et al.*, 2010; Sehgal *et al.*, 2011). The overall prevalence of trypanosomes exceeded 30% after

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microscopic examination of blood films; parasites of *T. avium* and *T. everetti* groups predominated in our samples (Valkiūnas *et al.*, 2011). One clearly distinguishable morphotype of a previously undescribed species of *Trypanosoma* was found during this study. Here we name and describe this parasite using data on the morphology of their hematozoic trypomastigotes, and partial sequences of the small subunit ribosomal RNA gene (SSU rDNA).

Materials and methods

Collection of blood samples. The blood samples used in this study were collected in Cameroon in July–November, 2005 and April 2006 (for more information see Bonneaud *et al.*, 2009; Chasar *et al.*, 2009) and in Ghana in July 2007 (see Loiseau *et al.*, 2010). Birds were caught with mist nets between daybreak (0600) and dusk (1700). They were banded, bled, and released. None of them was recaptured. Blood samples (50–100 µl) were collected by venipuncture of the brachial vein and stored in lysis buffer (10 mMTris-HCl, pH 8.0, 100 mM ethylene-diaminetetraacetic acid, 2% sodium dodecyl sulfate) for subsequent molecular analysis (Sehgal *et al.*, 2001). Two or 3 blood films were prepared from each bird. Blood films were air-dried within 5–10 sec after preparation. 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. During routine microscopy, 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 ´). Entire blood films from the type series were examined at low magnification.

Parasite morphology and statistical analysis. An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations, and to take measurements. The morphometric features studied (Table I) are those defined by Woo and Bartlett (1982) and Bennett, Siikamäki *et al.* (1994). The morphology of new *Trypanosoma* sp. was compared with the voucher specimens of *T. everetti, Trypanosoma ontarioensis, Trypanosoma anguiformis,* and *Trypanosoma polygranularis* (accession nos. 42151NS, 36221NS, 42022NS, and 36448NS, respectively) in the Collection of the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A *P*-value of 0.05 or less was considered significant.

DNA extraction, PCR, sequencing and analysis. Since samples were collected in remote field locations, culturing of the parasites was impractical (Sehgal *et al.*, 2001). To obtain total genomic DNA, blood was extracted following a DNeasy kit protocol (Qiagen®, Valencia, California), or the animal tissue protocol provided with the Wizard SV Genomic DNA Purification Kit (Promega Corporation, Madison, WI). The purified DNA was then used in a nested PCR protocol to amplify SSU rRNA DNA (Valkiūnas *et al.*, 2011). All PCR reactions were performed using *AccuPower* HotStart PCR PreMix (Bioneer, Alameda, California). The total reaction volume was 20 µl including primers, water, and template. The final concentration of each primer was 0.75 µM. The first set of primers was Tryp763 (5'-CATATGCTTGTTCAAGGAC-3') and Tryp 1016 (5'-CCCCATAATCTCCAATGGAC-3'). The thermal cycling profile started with an initial denaturation at 95° C for 5 min, followed by 5 cycles of at 95° for 1 min, 45° C for 30 sec, and 65° C for 1 min, followed by 35 cycles of 95° C for 1 min, 50° C for 30 sec, and 72° C for 1 min with a final extension at 65° C for 10 min. 2 µl of PCR product from the first reaction was used as template for the second PCR. The second set of primers was Tryp99 (5'-TCAATCAGACGTAATCTGCC-3') and Tryp957 (5'-CTGCTCCTTTGTTATCCCAT-3'). The cycling profile for the second PCR began with an initial denaturation at 96° C for 3 min, followed by 25 cycles of 96° C for 30 sec, 58° C for 1 min, 72° C for 30 sec, and a final extension at 72° C for 7 min. The expected fragment length was 770bp. We made efforts to obtain DNA sequences from the ITS (Internal Transcribed Spacer) regions using the PCR protocol from Cox *et al.* (2005), but attempts were unsuccessful for these samples. Products positive for infection were visualized on 1% agarose gels. Bidirectional sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Inc., Foster City, California). The new sequences were submitted to Genbank with the accession numbers (JX436209–JX436210).

The sequence divergence among lineages was calculated using the Jukes-Cantor model of substitution in which all substitutions were weighted equally in PAUP*4.0a1112 (Swofford, 2003). Genbank accession numbers for sequences of other avian trypanosomes used for comparisons are provided in Table II.

Results

Description of *Trypanosoma naviformis* n. sp.

(Figs. 1–5, Table 1).

Trypomastigotes easily found in thin blood films due to intensive staining and prominent kinetoplasts. Over 100 organisms seen in blood films; 21 non-deformed parasites measured (Table I). Parasites were small, spindle-shaped organisms with both posterior and anterior ends drawn out and symmetrically narrowed in relation to the parasite nucleus; these characteristics ascribe a 'boat'-like shape to the trypomastigotes and are a characteristic feature of this species. The most frequently seen trypomastigote type is shown in Figs. 1, 2. The cytoplasm stains unevenly blue, with clear poorly stained areas located around the nucleus, close to nucleus, and near kinetoplast; latter structure roundish (Figs. 3–5) or oval (Figs. 1, 2) in outline, prominent (Table I), stains densely purple, and situated close to posterior end of cell. Centrally placed nucleus large, markedly variable in shape, but more often oval. Azurophilic granules absent from the cytoplasm. Longitudinal striations not seen. Free flagellum originates just anterior to kinetoplast, poorly visible, so difficult to measure. Undulating membrane present, but undulations poorly visible and clearly seen in a few organisms (Figs. 2, 3, 5). No dividing forms seen. No information regarding pathogenicity in birds.

Taxonomic summary

Type host: Olive sunbird *Cyanomitra olivacea* (Passeriformes, Nectariniidae).

Additional hosts: Yellow-whiskered greenbul, *Andropadus latirostris* (Passeriformes, Pycnonotidae). Blood slide 42400NS, Collection of the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

DNA sequences: small subunit ribosomal RNA gene (SSU rDNA) lineages with GenBank™ accession JX436210.

Type locality: Agumatsa, (07°01.758′ N, 00°33.490′ E, 269 m above sea level), Ghana.

Distribution: In Ghana, *T. naviformis* has been reported at 3 sites, i.e., in the type locality and also in Abrafo and in Nkwanta (see Loiseau *et al.*, 2010 for detail description of these sites). This parasite is widespread in Cameroon where it was reported at 13 sites all over the country.

Site of infection: Blood plasma.

Prevalence: Overall prevalence in the olive sunbird was 79 of 380 (20.8%) in Ghana and Cameroon. In the type locality, the prevalence was 8 of 33 (24.2%).

Type specimens: Hapantotype (accession number 41736NS, over 20 trypomastigotes seen, *C. olivacea*, Agumatsa, 07°01.758′N, 00°33.490′E, Ghana, collected by G. Valkiūnas, 7 July 2007; GenBank JX436210) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius. Parahapantotypes were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania (accession no. 41735NS), in the U. S. National Parasite Collection, Beltsville, Maryland (USNPC 106222.01), and in the Queensland Museum, Queensland, Australia (G465639). Parasites are marked with circles on the hapantotype and parahapantotype slides.

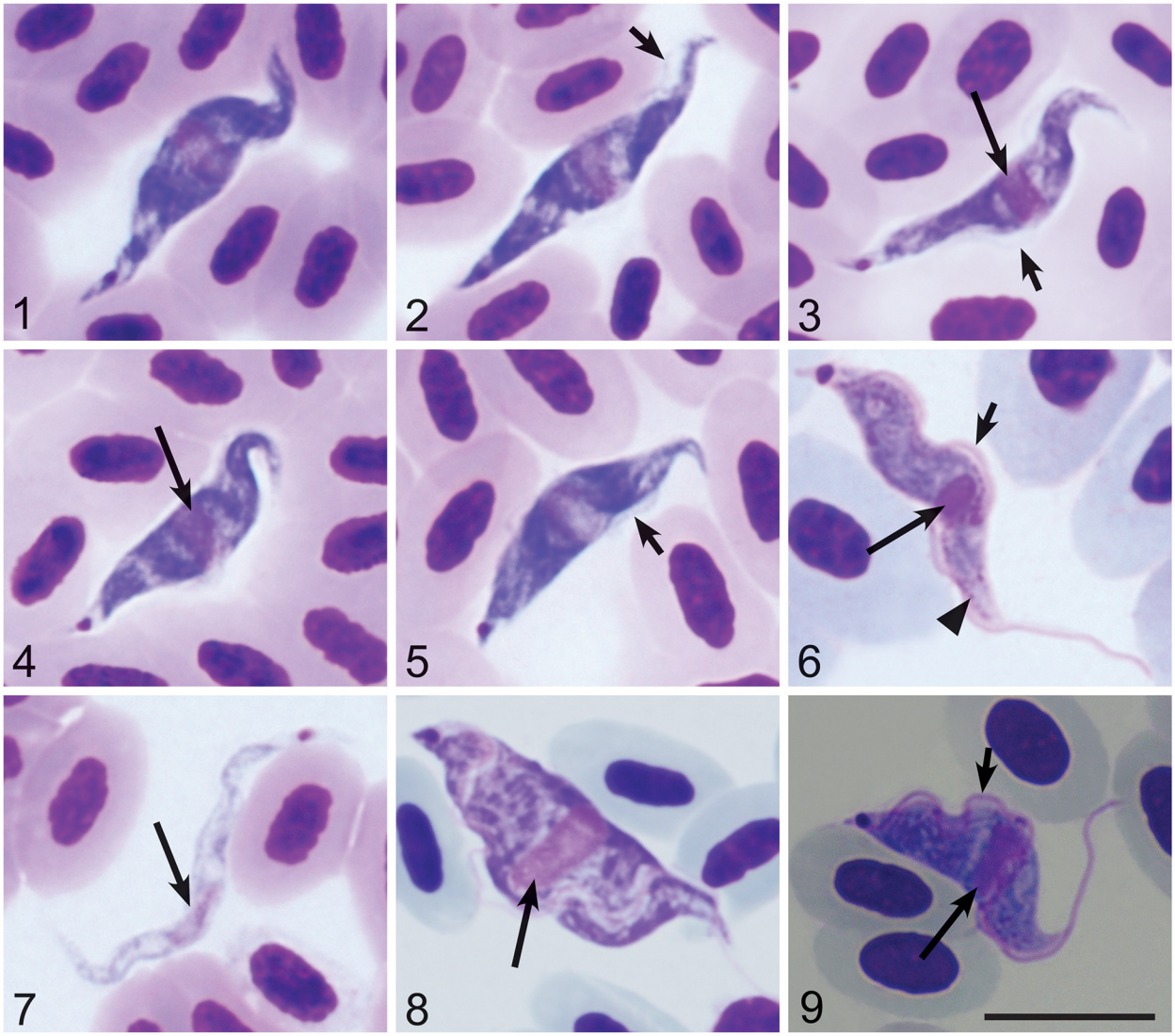
Additional material: Thirteen blood films (accession nos. 41625NS, 41657–41659NS, 41733NS, 41734NS) from the type host; 2 blood films (42399NS, 42400NS) from the additional host in Ghana; 5 blood films (48673NS–48677NS) from the type host in Cameroon were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. The samples of whole blood from the type and additional hosts (original field numbers are 271007, 271020, 271053, 271054, 271345, 251356, 251454, 251459, 251468, 251483) were deposited in the Conservation Genetics Resource Center at the University of California, Los Angeles, USA.

Etymology: The species name reflects the symmetrically pointed form of hematozoic trypomastigotes; this gives a 'boat'-like appearance of the parasite (see Fig. 2).

Remarks. Morphology and morphometric features of hematozoic trypomastigotes of *T. naviformis* in olive sunbird (Figs. 1–5, Table I) and yellow-whiskered greenbul (data not shown) are similar. There were no differences discernable among all morphometric parameters of trypomastigotes seen in these 2 avian hosts ($P > 0.1$ for all features reported in Table 1). Organisms reported in these bird species certainly belong to the same morphotype.

Trypanosoma naviformis belongs to the group of non-striated small avian trypanosomes (< 30 μm in length in average) with the kinetoplast situated close to the posterior end of the body. Numerous morphospecies of such trypanosomes have been described (Baker, 1976; Miltgen and Landau, 1982; Chandenier *et al.*, 1988; Valkiūnas *et*

al., 2011). The majority of the descriptions were based on single records of a few trypomastigotes, which were partially characterized, usually without designation of the type material. *Trypanosoma naviformis* is characterized by the following differential characters of hematozoic trypomastigotes: (1) nucleus is central in position; (2) organisms symmetrically narrowed from nucleus area toward both ends, resulting in a 'boat'-like shape (see Fig. 2); and (3) undulating membrane and flagellum are poorly visible (Figs. 3, 5). A combination of these readily distinguishable features are not characteristics of other described avian trypanosomes, which can be tentatively attributed to the group of small *Trypanosoma* spp., including fragmentally illustrated and described *T. laverani* (Novy and MacNeal, 1905), *T. calmettei* (Mathis and Léger, 1909), *T. caprimulgi minus* (Kerandel, 1913), *T. lagonostictae* (Marullaz, 1914), *T. cristatae* (Schwetz, 1931), *T. ixobrychi* (Mello, 1935), *T. turdoides* (Mello, 1935), *T. lobivanelli* (Mello, 1935; Mello, 1937), *T. oenae* (Sergent, 1941), and *T. fiadeiroi* (Tendeiro, 1947). The names of these parasites (excluding probably *T. calmettei*) should be attributed to the category *nomina dubia* until their re-description, as was suggested by Baker (1976).



FIGURES 1–9. Hematozoic trypomastigotes of *Trypanosoma naviformis* **n. sp.** from the Olive sunbird *Cyanomitra olivacea* (**1**–**5**), *Trypanosoma polygranularis* from the Latham's forest francolin *Francolinus lathami* (**6**), *Trypanosoma anguiformis* from the Olive sunbird *Cyanomitra olivacea* (**7**), *Trypanosoma everetti* from *Cyanomitra olivacea* (**8**), and *Trypanosoma ontarioensis* from the Yellow-whiskered greenbul *Andropadus latirostris* (**9**). Long arrows—nuclei of parasites. Short arrows—flagellum. Arrow heads—azurophilic granules. Giemsa-stained thin blood films. Bar = 10 µm.

Table I. Morphometry of hematozoic trypomastigotes (n = 21) of *Trypanosoma naviformis* from *Cyanomitra olivacea* in Ghana.

Feature*	Measurement (µm)†
AK	0.3-0.8 (0.6±0.1)
AN	5.5-11.3 (8.6±1.5)
AT	40.1-61.1 (50.3±6.1)
BW	3.2-5.3 (4.4±0.5)
KN	8.4-11.8 (9.5±0.9)
NA	9.4-13.8 (11.1±1.2)
PA	19.3-25.3 (22.5±1.6)
PK	1.0-2.9 (1.9±0.4)
PN	9.5-12.9 (11.2±0.8)
AN/AT	0.1-0.2 (0.16±0.03)
BW/PA‡	0.1-0.2 (0.19±0.02)
PK/PA	0.05-0.1 (0.08±0.02)
PN/KN	1.1-1.4 (1.2±0.1)
PN/NA	0.8-1.4 (1.0±0.1)
PN/PA	0.5-0.6 (0.5±0.03)

*Features: AK—area of kinetoplast, AN—area of nucleus, AT—area of trypomastigote, BW—width of body through centre of nucleus, KN—kinetoplast to centre of nucleus, NA—centre of nucleus to anterior end, PA—total length without free flagellum, PK—posterior end to kinetoplast, PN—posterior end to centre 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 indexes are calculated by division of the first feature by the second feature.

Table II. Sequence divergence (in percentage) between SSU rRNA lineages in identified *Trypanosoma* species. Numbers 1–4 represent different lineages of *T. naviformis.*

	Sample Number	GenBank	1.	2.	3.	4.	5.	6.	7.	8.
1.	251468T	JX436207	0							
2.	251468C	JX436208	0.14	0						
3.	251356	JX436209	0.14	0	0					
4.	271053/271345	JX436210	0.29	0.14	0.14	0				
5.	*T. anguiformis*	HQ992699	0.14	0.28	0.58	0.14	0			
6.	*T. avium*	AY099319	4.8	4.8	4.9	4.7	4.6	0		
7.	*T. benetti*	AJ223562	1.0	0.86	1.1	0.72	1.2	4.8	0	
8.	*T. corvi*	AY461665	5.3	5.1	5.4	5.0	5.0	2.9	4.5	0

Small trypomastigotes of *T. everetti* (Molyneux, 1973), *T. ontarioensis* (Woo and Bartlett, 1982) have been reported in numerous bird species, particularly passerines all over the world (Bennett, *et al.*, 1994; Bennett, *et al.*, 1994; Bishop and Bennett, 1992). *Trypanosoma anguiformis* and *T. polygranularis* were reported in Ghana and/or Cameroon, and the former parasitizes the olive sunbird in Ghana (Valkiūnas *et al.*, 2011). These 4 parasites are here compared with *T. naviformis.*

Trypomastigotes of *T. everetti, T. ontarioensis* and *T. polygranularis* are asymmetrically narrowed from their nuclear area to their ends (compare Figs. 1–3 and 6, 8, 9). They possess well-distinguished free flagella; thus, can be readily distinguished from *T. naviformis.* Additionally, 1) *T. anguiformis* possesses dumbbell-shaped nuclei, 2) *T. polygranularis* possesses a markedly off-center (anteriorly) located nucleus, numerous azurophilic granules that are arranged in a line following the undulating membrane, and a larger kinetoplast (area 2 times greater than in *T. naviformis,* $P < 0.01$), 3) *T. everetti* is an irregularly shaped parasite resembling a leaf or a kite in outline (Fig. 8) rather than the usual spindle shape associated with trypanosome morphology (Molyneux, 1973), which is not the case in *T. naviformis* (Figs. 1–5).

Sequence analysis. We obtained genetic sequences by using a nested PCR protocol to amplify SSU rRNA DNA, with a product of 770 base pairs (Valkiūnas *et al.*, 2011). Despite successful amplification, the sequence data lacked sufficient nucleotide variation to provide well-supported hierarchical structure for tree-based phylogenetic reconstructions. However, analysis of sequence data show that the trypanosomes differ from previously characterized avian trypanosomes (Table II). From the blood samples of African rainforest birds, we identified 2 unique lineages that represent *T. naviformis*. The sequence divergence within the 2 sequences representing this species averaged 0.14%. When comparing the sequences to previously identified avian trypanosomes, they are most closely related to *T. anguiformis* (Table II). Thus, both morphological and molecular evidence clearly identify *T. naviformis* as a novel small non-striated avian trypanosome.

Discussion

Recent work with avian trypanosomes, using microscropy in conjunction with molecular techniques, has resulted in a greater understanding of the diversity and prevalence of these relatively understudied parasites. Because of their morphological variability during trypomastigote stages, and generally light parasitemia, it is often difficult to identify morphospecies using solely microscopy. However, in certain cases, like the newly described species presented here, *T. naviformis*, microscopy has revealed intensive parasitemia with a parasite of consistent morphology in many individual avian hosts; this parasite clearly differs from other described avian trypanosomes. In conjunction with sequence data, it is apparent that this species is new. Moreover, it is ostensibly not a rare parasite, since it was found in the very common bird species, the olive sunbird and the yellow-whiskered bulbul at many sites in Ghana and Cameroon. In addition to parasitizing widespread African birds, *T. naviformis* was found on both sides of the Dahomey Gap, indicating that it is probably widespread in sub-Saharan Africa, and that it may predate the vicariant events that separated host populations on either side (Smith *et al.*, 2001).

Amplification of the SSU RNA gene described genetic variation in this unique morphospecies of *Trypanosoma*. Despite this information, a strongly supported phylogenetic tree could not be constructed. It will be useful to develop a better genetic marker in order to obtain more informative sequences from preserved blood, for further analyses. Progress has been made in characterizing new genes for identifying avian *Trypanosoma* spp.; however their applications are limited. Zidková *et al.* (2012) have demonstrated amplification of a more informative region of the SSU rDNA gene, and were able to construct well-supported phylogenetic trees from DNA obtained from cultured trypanosomes. In our efforts, the primers from that study were not successful in amplifying DNA from field-collected samples, most likely due to the interfering genetic material contained within avian nucleated red blood cells. During the present study, in order to obtain sequence data from alternative genes, we tested primers to obtain ITS (Internal Transcribed Spacer) region sequence of the ribosomal gene locus (Cox *et al.*, 2005). We were able to obtain only one sequence, Genbank accession no. JX443482, from an avian trypanosome sample found in the host *Andropadus nigriceps* (eastern mountain greenbul) collected in Uganda, (field number 23237) (Valkiūnas *et al.*, 2005). The sequence obtained from that sample was most closely related to *T. avium*, (GenBank accession number AY959322) with a sequence divergence of 3.34%. We were not able to obtain any other clean ITS sequences from *T. naviformis* described here nor from *T. anguiformis* (Valkiūnas *et al.*, 2011), most likely due to competing avian nuclear DNA. Optimizing the ITS protocol and obtaining more DNA sequences using high throughput sequencing from this and other species would be helpful in yielding an informative phylogenetic hypothesis.

Since the samples used in this study were isolated from birds in remote field locations in Africa, we did not attempt to culture the avian trypanosome described here. However we recommend that for future field expeditions with any intent of studying avian hematozoa, all attempts should be made to preserve blood samples for the propagation of trypanosomes in culture. It is clear that the morphology of cultured trypanosomes in many cases provides useful additional information for avian *Trypanosoma* spp. characterization, and sometimes appears to be more informative than what is found in blood smears from hosts (Zídková *et al.*, 2012). It should be mentioned that *in vitro* cultivation methods may also be limited in their application to taxonomy due to marked sensitivity and variation of morphology of parasite strains during development at different temperatures and media (Kirkpatrick and Terway-Thompson, 1986; Kučera, 1986). However, with cultured trypanosomes, it would be much more straightforward to obtain pure DNA, and thus facilitate molecular and phylogenetic analyses.

In the case presented here, *T. naviformis* was prevalent, parasitemia intensive, and readily distinct from other described trypanosomes. During microscopic identification in blood films, the small size of this trypanosome, coupled with its 'boat'-like shape and patterns of the cytoplasm staining (see the Description), provide valuable distinguishing features for the parasite (Figs. 1–5). How this parasite is transmitted is unknown, although based on available work, it is likely that ingestion of the vector may be important in its life cycle (Baker, 1976; Molyneux, 1977; Votýpka *et al.*, 2012; Zidková *et al.*, 2012). It is unlikely that birds would be discriminating in their choice of insects they can eat (or ingest incidentally), so this method of transmission may be an important contributor to the apparent lack of host specificity found among avian trypanosomes (Sehgal *et al.*, 2001).

Similarly, it is unclear why the prevalence and intensity of parasitemia of this parasite are high in certain populations using microscopic examination, which certainly underestimates true prevalence (Baker, 1976; Bennett, *et al.*, 1994); up to 24% of olive sunbirds were infected at the type locality, and tens of parasites were observed in many blood films. Whether there are age or seasonal effects on prevalence, or species behavioral or population effects, or circumstances permitting year-round transmission will be important areas of research. Our previous work has revealed that seasonal canopy moisture variability is an important predictor of avian trypanosome prevalence in the olive sunbird (Sehgal *et al.*, 2011). Thus, with global climate change, we would expect conditions to change regarding the ecology of this parasite, thus leading to changes in prevalence and perhaps host distributions. Although avian trypanosomes are thought to not cause significant pathology (but see Baker, 1976; Molyneux *et al.*, 1983), it will be important to continue in efforts to describe the parasites' diversity, mechanisms of adaptations leading to their low vertebrate-host specificity and the evolutionary and ecological conditions that contribute to their biogeography.

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