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TWO NEW *TRYPANOSOMA* SPECIES FROM AFRICAN BIRDS, WITH NOTES ON THE TAXONOMY OF AVIAN TRYPANOSOMES

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ABSTRACT: *Trypanosoma anguiformis* n. sp. and *Trypanosoma polygranularis* n. sp. are described from the African olive sunbird, *Cyanomitra olivacea*, and Latham's forest francolin, *Francolinus lathami*, respectively, based on the morphology of their hematozoic trypomastigotes and partial sequences of the small subunit ribosomal RNA gene. Both new species belong to the group of small non-striated avian trypanosomes (<30 μm in length on average) with the kinetoplast situated close to the posterior end of the body. *Trypanosoma anguiformis* can be readily distinguished from other small avian trypanosomes due to its markedly attenuated (snake-shaped) form of the hematozoic trypomastigotes and the dumbbell-shaped nucleus of the parasite. *Trypanosoma polygranularis* is readily distinguishable due to the markedly off-center (anteriorly) located nucleus, numerous azurophilic granules that are arranged in a line following the undulating membrane, and the large kinetoplast (with an area up to 1.7 μm^2 [1.1 μm^2 on average]). Illustrations of hematozoic trypomastigotes of the new species are given, and DNA lineages associated with these parasites are reported. The current situation in species taxonomy of avian trypanosomes is discussed. We call for the redescription of valid species of avian trypanosomes from their type vertebrate hosts and type localities by using morphological and polymerase chain reaction-based techniques as an initial essential step towards revising the species composition of avian trypanosomes and reconstructing the taxonomy of these organisms.

Species of avian *Trypanosoma* (Kinetoplastida: Trypanosomatidae) are surprisingly successful blood parasites that are widespread and prevalent in birds throughout the world (Bishop and Bennett, 1992). They are perfectly adapted for transmission in various terrestrial ecosystems, including oceanic islands and countries with cold climates where avian hosts and blood-sucking arthropod vectors are present (Baker, 1976; Bennett, Siikamäki et al., 1994; Holmstad et al., 2003; Valkiūnas, 2005; Deviche et al., 2010). Avian trypanosomes are transmitted by wide variety of blood-sucking arthropods belonging to the Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae, and Dermanyssidae (Baker, 1976; Molyneux, 1977; Miltgen and Landau, 1982; Votýpka and Svobodová, 2004). The same strains and lineages of trypanosomes can successfully develop in numerous species of phylogenetically distant avian hosts belonging to different families and even orders (Bennett, 1961; Apanius, 1991; Sehgal et al., 2001). The mechanisms of broad ecological plasticity of avian trypanosomes remain insufficiently investigated. Due to regular seasonal transcontinental migrations, this host–parasite system might provide new insight to study mechanisms of ecological plasticity, specificity, and speciation in parasitic kinetoplastids and thus warrants the attention of parasitologists and evolutionary biologists.

In spite of extensive research over a 120-yr period, the taxonomy of avian trypanosomes has been surprisingly poorly developed; this is an obstacle for further fundamental studies in this field of wildlife parasitology. More than 100 species of avian *Trypanosoma* have been named and described, mainly based on morphological features of hematozoic trypomastigotes, i.e., so-called bloodstream forms (Baker, 1976; Bennett, Earlé et al., 1994). Many *Trypanosoma* species were insufficiently illustrated and described in early publications, making it difficult or even impossible to use the majority of original descriptions for species identification and morphological comparison. In addition, early original descriptions are based on the belief that the parasites are strictly vertebrate-host specific, frequently on the level of species of birds, but this was shown to be incorrect by later experimental and molecular studies (Bennett, 1961, 1970; Chatterjee and Ray, 1971; Fallis et al., 1973; Molyneux and Gordon, 1975; Sehgal et al., 2001; Votýpka and Svobodová, 2004; but see also Molyneux, 1973a). The majority of species names of avian trypanosomes are provisional and are of doubtful application and thus should be considered as nomina dubia and necessitate future clarification; this is the unanimous opinion in the current taxonomy (see Baker, 1976; Apanius, 1991; Bennett, Siikamäki et al., 1994; Votýpka et al., 2002).

Morphological evidence suggests the existence of numerous species of avian trypanosomes (Baker, 1976; Kučera, 1986; Bennett, Earlé et al., 1994); this is in accordance with the current limited information on the ultrastructure, biochemistry, immunology, and molecular phylogeny of avian trypanosomes (Molyneux and Gordon, 1975; Kirkpatrick et al., 1986; Sehgal et al., 2001; Votýpka et al., 2002, 2004; Sehgal et al., 2006). However, many existing species and the species limits remain unclear even in relatively well-investigated host–parasite systems. For example, large trypomastigotes of *Trypanosoma avium* and *Trypanosoma corvi* (both are indistinguishable using light microscopy and usually are attributed to the *T. avium* group) parasitize the rook, *Corvus frugilegus*, and are sympatric in some European countries (Baker, 1976; Votýpka et al., 2004); they are examples of sibling species in the *T. avium* group that might consist of numerous parasites species (Bennett, Siikamäki et al., 1994). These organisms can be distinguished based on DNA sequences, the ultrastructure of their kinetoplast, vector preference, and some other features. It is possible that complexes of related (sibling) species are described under the many available *Trypanosoma* names (Baker, 1976; Bennett, Earlé et al., 1994).

Experimental studies show that many *Trypanosoma* species can be readily distinguished using morphological and morphometric characters during co-infections (Molyneux and Gordon, 1975). Being morphologically variable (particularly during development in vectors and in the early morphogenesis of metacyclic forms in birds), fully grown hematozoic trypomastigotes are relatively conservative in their gross morphology, i.e., body shape, limits of body size, position and morphology of kinetoplast, morphological features of the flagellum and undulating membrane, and morphometric indexes. Importantly, the main morphological

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features of certain species remain the same during their development in different experimentally infected avian hosts (Baker, 1956, 1976; Bennett, 1961; Chatterjee and Ray, 1971; Fallis et al., 1973; Molyneux, 1973a; Votýpka and Svobodová, 2004). For example, hematozoic trypomastigotes of *T. everetti* never assume forms typical of *T. avium* or *Trypanosoma bouffardi* during single and simultaneous experimental infections and, on the contrary, remain identifiable (Molyneux and Gordon, 1975). These experiments show that, despite some morphometric variations during development of the same *Trypanosoma* strains in different avian hosts, the gross morphological features of hematozoic trypomastigotes are relatively stable and thus can be used to recognize new readily distinguishable morphotypes that might be new parasite species or groups of species. Identification of such complexes of species is an important step in developing 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, 1973b).

During recent studies on the effects of deforestation on the prevalence of blood pathogens in African rain forest birds (see Bonneaud et al., 2009; Chasar et al., 2009; Iezhova et al., 2010; Loiseau et al., 2010; Sehgal et al., 2010), large numbers of blood samples were collected from >100 species of birds. The overall prevalence of trypanosomes exceeded 30% after microscopic examination of blood films; parasites of *T. avium* and *T. everetti* groups predominated in our samples (Valkiūnas, pers. obs.). Two clearly distinguishable morphotypes of previously undescribed species of *Trypanosoma* were found during this study. These parasites are named and described here by using data on the morphology of their hematozoic trypomastigotes and partial sequences of the small subunit ribosomal RNA (SSU rRNA) gene. We also discuss the current situation in the species taxonomy of avian trypanosomes.

MATERIAL AND METHODS

Collection of blood samples and extraction of DNA

The blood samples used in this study were collected in Bobo Camp, southeastern Cameroon, in July 2005 (for more information, see Bonneaud et al., 2009) and in Abrafo and Agumatsa, Ghana, in July 2007 (see Loiseau et al., 2010). Birds were caught with mist nets between daybreak (0600 hr) and dusk (1700 hr). They were ringed, 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 mM Tris-HCl, pH 8.0, 100 mM ethylene-diaminetetraacetic acid, and 2% sodium dodecyl sulfate) for subsequent molecular analysis (Sehgal et al., 2001). Two, 3, or 4 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. During routine microscopy, blood films were examined for 10–15 min by using 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

A BX61 light microscope (Olympus, Tokyo, Japan) equipped with a DP70 digital camera (Olympus) 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 morphologies of new *Trypanosoma* spp. were compared with the voucher specimens of *T. everetti* and *Trypanosoma ontarioensis* (accessions 42151NS, 41691NS, and 36221NS, 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 was considered significant.

TABLE I. Morphometry of hematozoic trypomastigotes of 2 new *Trypanosoma* species from African birds.

	Measurement (µm)†	
Feature*	*T. anguiformis* (n = 21)	*T. polygranularis* (n = 25)
AK	0.3–0.7 (0.6 ± 0.1)	0.6–1.7 (1.1 ± 0.3)
AN	3.1–7.4 (5.4 ± 1.3)	5.0–10.8 (7.3 ± 1.7)
AT	31.1–47.9 (39.2 ± 4.2)	41.7–67.7 (51.7 ± 6.4)
BW	1.5–2.6 (2.0 ± 0.3)	2.7–4.4 (3.5 ± 0.4)
FF	4.1–4.6 (4.3 ± 0.3)‡	7.7–11.6 (10.0 ± 1.0)
KN	10.8–16.2 (14.1 ± 1.2)	10.1–13.2 (11.6 ± 0.7)
NA	9.8–13.4 (11.8 ± 1.0)	4.7–9.7 (7.3 ± 1.0)
PA	25.2–30.8 (28.6 ± 1.5)	17.0–23.0 (20.6 ± 1.2)
PK	1.4–3.1 (2.3 ± 0.5)	1.2–2.6 (1.8 ± 0.3)
PN	12.8–17.8 (16.2 ± 1.2)	10.9–16.3 (13.3 ± 1.0)
AN/AT§	0.1–0.2 (0.1 ± 0.03)	0.1–0.2 (0.1 ± 0.03)
BW/PA	0.05–0.09 (0.07 ± 0.01)	0.1–0.2 (0.2 ± 0.02)
PK/PA	0.05–0.1 (0.08 ± 0.01)	0.06–0.11 (0.09 ± 0.01)
PN/KN	1.0–1.2 (1.1 ± 0.1)	1.0–1.3 (1.1 ± 0.07)
PN/NA	1.1–1.8 (1.4 ± 0.2)	1.3–2.7 (1.9 ± 0.3)
PN/PA	0.5–0.6 (0.6 ± 0.03)	0.5–0.8 (0.7 ± 0.05)

* 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 SD.
‡ Number of measurements, n = 2.
§ This index and indexes given below are calculated by division of the first feature by the second feature.

Polymerase chain reaction (PCR), sequencing, and analysis

Because 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). Extracted DNA was used in nested PCR reactions to amplify SSU rRNA fragments according to a protocol modified from Sehgal et al. (2001). New primers for a second round of the nested PCR reaction were designed for this study to increase the fragments size of the sequences from 326 to 770 bp. The first set of primers was Tryp763 (5′-CATATGCTTGTTTCAAGGAC-3′ and Tryp1016 (5′-CCCCATAAT-CTCCAATGGAC-3′) and the second set was Tryp99 (5′-TCAATCA-GACGTAATCTGCC-3′ and Tryp957 (5′-CTGCTCCTTTGT TATCCC AT-3′). The cycling profile conditions were as follows: initial denaturation at 95 C for 5 min, followed by 5 cycles 95 C 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, 72 C for 1 min, and then a final extension at 65 C for 10 min. We used 2 µl of the first PCR reaction as the template for the second PCR. The reaction conditions using the second primer set were as follows: 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 then a final extension at 72 C for 7 min. Bidirectional sequencing of the PCR fragments was performed in an ABI Prism 377 automated sequencer (Applied Biosystems, Inc., Foster City, California).

New sequences are deposited in GenBank™, with the accessions HQ992697–HQ992700 and HQ992702. For comparison of trypanosome sequences, published sequences for the SSU rRNA gene were obtained from GenBank by using the following accessions: AY491764 (*Trypanosoma blanchardi*), AY099319 (*Trypanosoma avium*), AJ223562 (*Trypanosoma bennetti*), AF306776 (*Trypanosoma brucei*), AY461665 (*Trypanosoma*

corvi), FJ900240 (*Trypanosoma cruzi*), AJ005278 (*Trypanosoma grayi*), FJ649479 (*Trypanosoma irwini*), AF119808 (*Trypanosoma mega*), AJ009158 (*Trypanosoma microti*), AF119810 (*Trypanosoma ranarum*), AJ012417 (*Trypanosoma rangeli*), AJ009161 (*Trypanosoma rotatorium*), AB007814 (*Trypanosoma theileri*), and AJ005279 (*Trypanosoma varani*). These sequences were identified using an NCBI BLAST-search. Sequences were aligned using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, Michigan). We conducted phylogenetic analyses of the sequences by using parsimony, and maximum-likelihood techniques implemented in PAUP*4.0b10 (Swofford, 2003). 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 *Trypanosoma* spp. Due to this lack of phylogenetic resolution, we therefore interpreted our results primarily by determining genetic divergence between recorded lineages. Pairwise base differences among lineages were determined using PAUP*4.0b10 (Swofford, 2003).

RESULTS

DESCRIPTION

Trypanosoma anguiformis n. sp.

(Figs. 1–3; Table I)

Diagnosis: Trypomastigotes relatively difficult to see in thin blood films due to pale staining and attenuated shape of organisms; >40 seen in infected birds, with numerous parasites recorded in each blood film. Parasites small, snake-shaped, markedly attenuated organisms (average width of body through center of nucleus is 2 μm), with both posterior and anterior ends markedly drawn out and pointed. Cytoplasm stains unevenly pale blue, with clear poorly stained areas located around nucleus and near kinetoplast; latter structure oval (Figs. 1, 2) or rod-like (Fig. 3) in outline, prominent (Table I), stains densely purple, and situated close to posterior end of cell. Centrally placed nucleus large, markedly variable in shape, usually elongated and never assuming roundish form, frequently dumbbell-shaped due to clearly visible 2 clumps of chromatin; this form of nucleus a distinctive character of this parasite (Figs. 2, 3). Longitudinal striations not seen. Free flagellum originates just anterior to kinetoplast, poorly visible, and thus difficult to measure (Table I). Undulating membrane present, but undulations poorly visible and clearly seen in few organisms (Fig. 1). No dividing forms seen. No information regarding pathogenicity in birds.

Taxonomic summary

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

Additional hosts: Unknown.

DNA sequences: Four SSU rRNA gene lineages with GenBank accessions HQ992698–HQ992700 and HQ992702.

Type locality: Abrafo (05°21.171′N, 01°23.406′E; 170 m above sea level), Ghana.

Distribution: This parasite has been reported only in Ghana where it was found at 2 sites, i.e., in the type locality and in Agumatsa (07°01.758′N, 00°33.490′E; 269 m above sea level).

Site of infection: Blood plasma.

Prevalence: Overall prevalence in the olive sunbird was 6 of 104 (5.8%) in Ghana. In the type locality, the prevalence was 4 of 30 (13.3%).

Type specimens: Hapantotype (accession 42022NS, 9 trypomastigotes seen, *C. olivacea*, Abrafo, Ghana, 05°21.171′N, 01°23.406′E, collected by G. Valkiūnas, 13 July 2007) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (42021NS and USNPC 104367.01, USNPC 104368.01, other data as for the hapantotype) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, and in the U.S. National Parasite Collection, Beltsville, Maryland, respectively. Parasites are marked with circles on the hapantotype and parahapantotype slides.

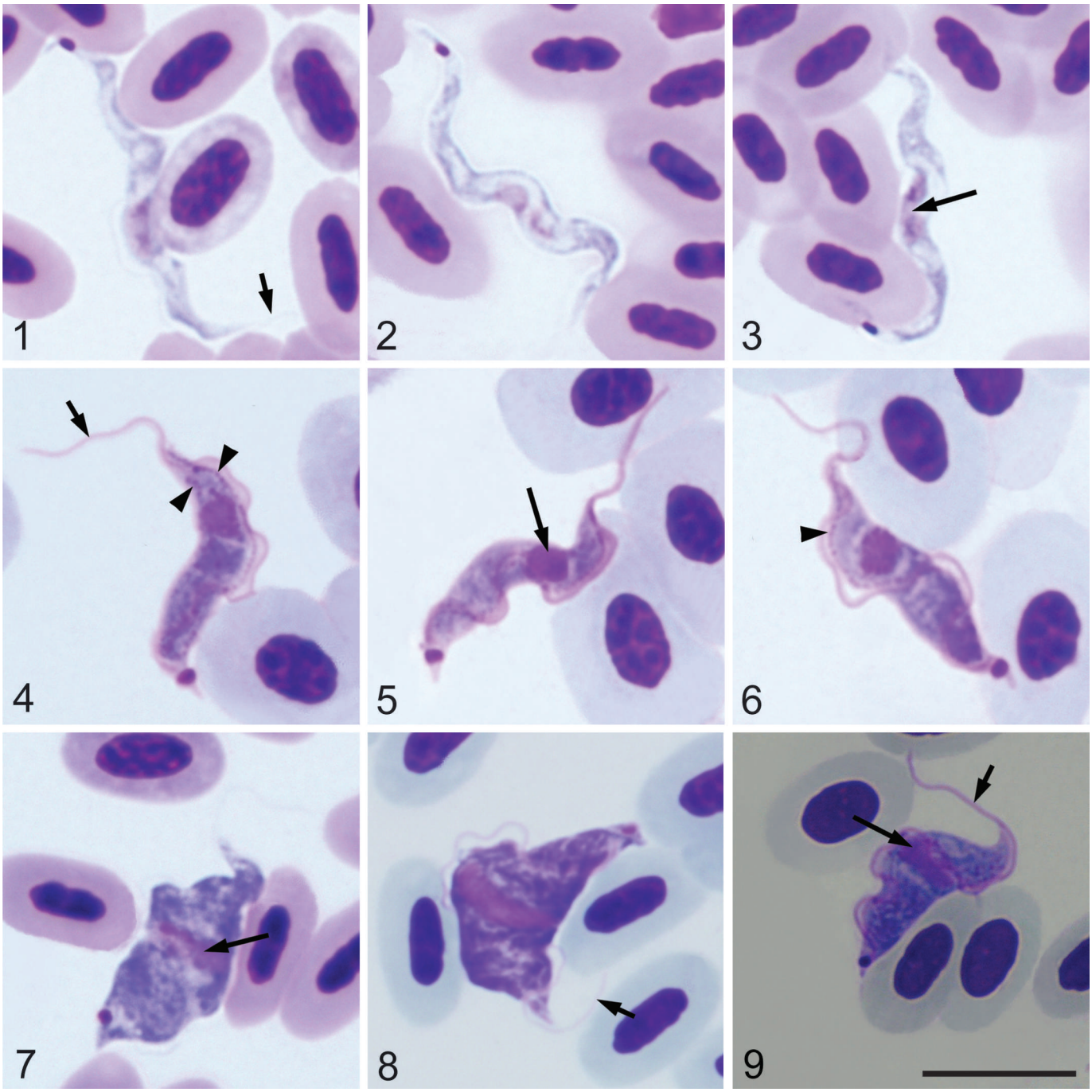
Additional material: Eight blood films (accessions 41842NS, 41843NS, 41997NS, 41998NS, 42100NS, 42101NS, 42162NS, and 42163 NS from the type host in Ghana) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. The samples of whole blood from the type host (original field numbers are 27-1096, 27-1166, 27-1168, 27-1234) were deposited in the Conservation Genetics Resource Center at the University of California, Los Angeles, California.

Etymology: The species name reflects the markedly attenuated snake-like form of hematozoic trypomastigotes.

Remarks

Trypanosoma anguiformis belongs to the group of non-striated small avian trypanosomes (<30 μm in length on average) with the kinetoplast situated close to the posterior end of the body. More than 15 species of such trypanosomes have been described (Baker, 1976; Miltgen and Landau, 1982; Chandenier et al., 1988); the majority of the descriptions were based on single records of a few trypomastigotes that were partially characterized, usually without designation of the type material. *Trypanosoma anguiformis* is characterized by the following differential characters of hematozoic trypomastigotes: (1) organisms are markedly attenuated (snake-shaped), (2) nucleus is dumbbell-shaped, and (3) undulating membrane and flagellum are poorly visible (Figs. 2, 3). A combination of these readily distinguishable features are not characteristics of other avian trypanosomes that can be tentatively attributed to the group of small *Trypanosoma* spp., including fragmentally illustrated and described *Trypanosoma laverani* (Novy and MacNeal, 1905), *Trypanosoma calmettei* (Mathis and Léger, 1909), *Trypanosoma caprimulgi minus* (Kerandel, 1913), *Trypanosoma lagonostictae* (Marullaz, 1914), *Trypanosoma cristatae* (Schwetz, 1931), *Trypanosoma ixobrychi* (de Mello, 1935), *Trypanosoma turdoides* (de Mello, 1935), *Trypanosoma lobivanelli* (de Mello, 1937), *Trypanosoma oenae* (Sergent, 1941), and *Trypanosoma fiadeiroi* (Tendeiro, 1947). Names of these parasites (excluding probably *T. calmettei*) should be attributed to the category nomina dubia until their redescription, as was suggested by Baker (1976).

Small trypomastigotes of *T. everetti* (Molyneux, 1973b) and *T. ontarioensis* (Woo and Bartlett, 1982) have been frequently reported in numerous bird species, particularly passerines all over the world (Bishop and Bennett, 1992; Bennett, Earlé et al., 1994; Bennett, Siikamäki et al., 1994), so should be distinguished from *T. anguiformis*. Trypomastigotes of both *T. everetti* and *T. ontarioensis* are much less attenuated than *T. anguiformis* (compare Figs. 1–3 and 7–9); they possess well-distinguished free flagella and do not possess dumbbell-shaped nuclei, thus they can be readily distinguished from *T. anguiformis*. In addition, *T. everetti* is an irregularly shaped parasite resembling a leaf or a kite in outline (Figs. 7, 8) rather than the usual spindle shape associated with trypanosome morphology (Molyneux, 1973b), which is not the case in *T. ontarioensis* (Fig. 9).



FIGURES 1–9. Hematozoic trypomastigotes of *Trypanosoma anguiformis* n. sp. from the olive sunbird, *Cyanomitra olivacea* (**1–3**), *Trypanosoma polygranularis* n. sp. from the Latham's forest francolin, *Francolinus lathami* (**4–6**), *Trypanosoma everetti* from olive sunbird (**7–8**), and *Trypanosoma ontarioensis* from the yellow-whiskered greenbul, *Andropadus latirostris* (**9**). Long arrows indicate nuclei of parasites. Short arrows indicate flagellum. Arrowheads indicate azurophilic granules. Giemsa-stained thin blood films. Bar = 10 μm.

***Trypanosoma polygranularis* n. sp.**

(Figs. 4–6; Table I)

Diagnosis: Trypomastigotes easily found in thin blood films due to intensive staining, well-distinguishable flagellum and large kinetoplast. More than 60 organisms seen in blood films; 25 non-deformed parasites investigated and measured (Table I). Parasites small, spindle-shaped organisms with both posterior and anterior ends drawn out and pointed. Cytoplasm stains blue, homogenous in appearance, possesses a few lightly stained spaces and numerous minute roundish azurophilic granules; a majority of the latter structures are arranged linearly, which follows undulating membrane (Fig. 6), but some also seen separately in cytoplasm (Fig. 4). Kinetoplast large (Table I), prominent, usually roundish in outline, occasionally oval, stains densely purple; situated close to posterior end of cell (Figs. 4–6). Nucleus compact, usually roundish, located clearly off-center; distance of

nucleus to posterior end approximately 2 times greater than to anterior end (Table I). Longitudinal striations not seen. Free flagellum originates just anterior to kinetoplast, densely stained, and well distinguishable. Undulating membrane conspicuous, with well pronounced undulations; undulation number varies (Figs. 4–6) but usually 4–5 (on average, 4.7 ± 0.5). No dividing forms seen. No information regarding pathogenicity in birds.

Taxonomic summary

Type host: Latham's forest francolin, *Francolinus lathami* Hartlaub (Galliformes: Phasianidae).

Additional hosts: Unknown.

DNA sequences: SSU rRNA gene lineages with GenBank accession HQ992697.

Type locality: Bobo Camp, Cameroon (2°39′17″N, 13°28′ 16″E; 170 m above sea level).

Distribution: This parasite has been reported only at the type locality so far.

Site of infection: Blood plasma.

Prevalence: One of 1 investigated the Latham's francolin was infected at the type locality.

Type specimens: Hapantotype (accession 36448NS, 17 trypomastigotes seen, *F. lathami*, Bobo Camp, Cameroon, 2°39′17″N, 13°28′16″E, collected by G. Valkiūnas, 19 July 2005) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (36449NS and USNPC 104369.01, USNPC 104370.00), other data as for the hapantotype) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, and in the U.S. National Parasite Collection, Beltsville, Maryland, respectively. Parasites are marked with circles on the hapantotype and parahapantotype slides.

Additional material: One sample of whole blood from the type host (original field number is 25-433) was deposited in the Conservation Genetics Resource Center at the University of California, Los Angeles, California.

Etymology: The species name reflects the presence of numerous minute azurophilic cytoplasmic granules, which are arranged as a line following the undulating membrane in trypomastigotes.

Remarks

Trypanosoma polygranularis can be readily distinguished from *T. anguiformis* due to the 3 characters of the latter parasite mentioned in the Remarks to that species, as well as both the conspicuous flagellum and undulating membrane. In addition, the area of the kinetoplast of *T. polygranularis* is approximately 2 times greater ($P < 0.001$) than in *T. anguiformis* (Table I), and the kinetoplast of the former species never assumes a rod-like form that is a frequent case in *T. anguiformis* (compare Figs. 3 and 6).

Trypanosoma polygranularis belongs to the group of small avian trypanosomes, as does *T. anguiformis*; it should be compared with the same species of parasites (see Remarks to *T. anguiformis*). *Trypanosoma polygranularis* is characterized by the following differential characters (Figs. 4–6): (1) nucleus is markedly off-center in position and located closer to the anterior end of the organism, (2) the cytoplasm possesses numerous azurophilic granules that are arranged as a line following the undulating membrane, (3) kinetoplast is large (Table I), and (4) the free flagellum is long and conspicuous (Table I). A combination of these readily distinguishable features is not characteristic of other avian trypanosomes that can be tentatively attributed to the group of small *Trypanosoma* spp. (see Remarks to *T. anguiformis*), including *T. everetti* and *T. ontarioensis* (Figs. 7–9).

Trypanosoma polygranularis should be distinguished from *T. calmettei* that was originally described from domestic chickens in Tonkin, Vietnam (Mathis and Léger, 1909). Bennett, Earlé et al. (1994) attributed to *T. calmettei* a single infection with only 2 trypomastigotes that were detected in the natal francolin, *Francolinus natalensis*, in Zimbabwe; these authors believed that the parasites are similar to *T. calmettei*, but they provided neither a description nor illustrations. *Trypanosoma polygranularis* can be distinguished from *T. calmettei* due to (1) greater size of kinetoplast (not measured but illustrated in the originals description of *T. calmettei* by Mathis and Léger, 1909), (2) marked of-center location of nucleus in trypomastigotes, and (3) numerous azurophilic granules arranged as a line in the cytoplasm.

Phylogenetic analysis

We used a nested PCR protocol to obtain SSU rRNA DNA sequences of 770 bp. However, even with this sequence length, we were unable to construct informative phylogenetic trees with high support for branch nodes. We obtained 4 closely related sequences from samples of *T. anguiformis*. The sequence divergence within the 4 sequences representing this species averaged 0.5%. The sequence data for *T. anguiformis* showed high similarity to *T. bennetti*, with an average sequence divergence of 0.8% among sequences representing these 2 species.

We obtained sequence data from 1 sample of *T. polygranularis*. The sequence of this species is most closely related to *T. bennetti*, with a sequence divergence of 2.0%. It also is closely related to *T. anguiformis*, with an average sequence divergence of 2.8%.

The *T. polygranularis* lineage differed from the published sequences of *T. avium* and *T. corvi* and *T. everetti* by approximately 5.0, 6.0, and 7.0%, respectively. The average sequence divergence between lineages of *T. anguiformis* and the same avian parasites was 4.5, 5.0, and 7.0%, respectively.

DISCUSSION

Numerous field studies on avian trypanosomes are based on the microscopic examination of blood films. Due to investigations in remote areas, biochemical, immunological, and in vitro cultivation methods have been applied to study only a few species of avian trypanosomes (see Molyneux, 1973a; Molyneux and Gordon, 1975; Baker, 1976; Woo and Bartlett, 1982; Kirkpatrick et al., 1986; Kučera, 1986; Apanius, 1991; Nandi and Bennett, 1994), so they currently cannot be used for broad taxonomic work; accumulation of such information is needed. DNA sequence-based data on avian trypanosomes also remain scarce (Sehgal et al., 2001; Votýpka et al., 2004; Sehgal et al., 2006, 2010).

In spite of clear limitations of microscopy, particularly the low sensitivity of microscopy in detecting infections, and the remarkable morphological variations of the parasites in vectors and at early stages of their development in avian hosts (Baker, 1956; Bennett, 1962; Baker, 1976; Kirkpatrick et al., 1986; Apanius, 1991), it is still reasonable to use relatively cheap microscopic techniques in determining patterns of distribution of

the main morphotypes of hematozoic trypomastigotes in wildlife. Using microscopy initially will help to direct the application of advanced and expensive technologies in investigations of these organisms in wildlife, particularly in remote tropical areas. The present study adds 2 new morphospecies (Figs. 1–6) and contributes to the diversity of hematozoic forms of avian *Trypanosoma* spp.

The molecular work here further underscores the limitations of using the SSU rRNA gene as a marker for inferring phylogenetic relationships among avian trypanosomes (Sehgal et al., 2001). Sequence data can be used as a bar code to help identify the numerous species, but due to the overall similarity in sequence data, it has been insufficient in supporting evolutionary relationships. This finding is similar to results of Votýpka et al. (2002), who found that due to a polytomy, the relationships among *T. avium*, *T. bennetti*, *T. scelopori*, *T. varani*, and *T. grayi* could not be resolved using the 18S rRNA gene. Further work showed that *T. avium* clearly differs from *T. corvi* (Votýpka et al., 2004), but the relationship of *T. bennetti* and the newly described *T. anguiformes* and *T. polygranularis* to other avian trypanosomes remains unresolved. Future work is needed, perhaps with whole genome analyses, to clearly infer the phylogenetic relationships of these parasites.

Our molecular analysis of the SSU rRNA gene shows that both *T. anguiformis* and *T. polygranularis* are closely related to *T. bennetti*. All 3 species belong to a group of small avian trypanosomes. Unfortunately, hematozoic trypomastigotes were not included in the original description of *T. bennetti* (Kirkpatrick et al., 1986), and their morphology remains unknown. Morphological comparison of these parasites is currently impossible, but the data presented here will aid in distinguishing these organisms based on their DNA sequences.

Information on species taxonomy and valid names of avian trypanosomes was discussed by Baker (1976) in detail; the majority of his conclusions regarding species taxonomy of avian *Trypanosoma* remain valid, with minor modifications concerning species tentatively belonging to the *T. avium* group. Recent studies on *T. avium* and *T. corvi* have shown that both these parasites can infect the same avian hosts and can be transmitted sympatrically (Votýpka et al., 2004). Thus, the host range and the geographical distribution can hardly be used in recognition of these morphologically similar representatives of *T. avium* group. This discovery's implications further complicate the identification and classification of avian trypanosomes. Before additional information is available, it is still reasonable to classify avian trypanosomes in species groups, as suggested by Baker (1976) and Bennett, Earlé et al. (1994).

Progress in the taxonomy of avian trypanosomes remains extremely slow (see Baker, 1976; Sehgal et al., 2006). That is not only because of (1) incomplete early original descriptions; (2) difficulties in application biochemical and culture techniques in comparative studies of wildlife parasites, particularly in remote areas (Kirkpatrick et al., 1986; Kučera, 1986; Apanius, 1991; Votýpka et al., 2004); and (3) difficulties in use of some PCR-based techniques during light *Trypanosoma* spp. infections (Sehgal et al., 2001) but also because of lack of the type material and also DNA sequence information for type specimens of the majority of described species. The latter hinders comparative taxonomic studies because of questionable identification of similar morphotypes recorded in different avian hosts and different study sites. Redescription of valid species from their type vertebrate hosts and type localities by using morphological and PCR-based techniques, as well as the deposition of the type material in museums, are essential for the revision of the species composition of avian trypanosomes and the reconstruction of the taxonomy of these organisms. This is a particularly alarming issue for the *T. avium* group, parasites that apparently infect a broad range of avian hosts and might be of global distribution (Bennett, 1961; Bennett, Siikamäki et al., 1994). Type material has been designated for *T. everetti* (Molyneux, 1973b), *T. bennetti* (Kirkpatrick et al., 1986), *T. gallinarum* (Sehgal et al., 2006), *T. corvi* (Nandi and Bennett, 1994), and a few recently described parasites (Miltgen and Landau, 1982; Chandenier et al., 1988), including the 2 new species described here, i.e., *T. anguiformis* and *T. polygranularis*.

Taxonomy and systematics are essential in understanding the natural world, but they remain poorly developed for avian trypanosomes; this is an obstacle to their use in evolutionary biology studies. The number of publications on avian trypanosomes is negligible in comparison with that of the *Trypanosoma* spp. of mammals (<0.1% according to the Scopus database on 28 January 2011). This is understandable because of negligible economic importance of these parasitic organisms (but see also Molyneux et al., 1983). However, avian trypanosomes are characterized by many unique properties (Baker, 1976; Molyneux, 1977; Votýpka et al., 2004); they are widespread, diverse, and inhabit a fascinating group of vertebrate hosts. They thus may provide innovative insights in understanding the evolutionary biology of all trypanosomes (Apanius, 1991). We call for the need of taxonomic and systematic studies on avian *Trypanosoma*.

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