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PROTOZOOLOGY - ORIGINAL PAPER

Morphological and molecular characterization of avian trypanosomes in raptors from Thailand

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Abstract

From September 2012 to May 2018, blood samples from 364 raptors (mostly adults) were collected and screened for trypanosomes and haemosporidians by microscopic examination and nested polymerase chain reactions (PCR). *Trypanosoma* spp. were identified in 15 birds from eight different species. Light microscopy revealed 14 cases of infection with *Trypanosoma* cf. *corvi*, including one each in black-shouldered kite (*Elanus caeruleus*, $n = 49$), Brahminy kite (*Haliastur indus*, $n = 50$), and spotted owlet (SO, *Athene brama*, $n = 27$); two mountain hawk-eagles (*Spizaetus nipalensis*, $n = 3$); and three each in Asian barred owlets (ABO, *Glaucidium cuculoides*, $n = 27$), barn owls (BO, *Tyto alba*, $n = 65$) and collared scops owls (CSO, *Otus lettia*, $n = 41$). In addition, one case of infection with *T. avium* was identified in an oriental scops owl (OSO, *Otus sunia*, $n = 2$). All infected raptors showed very low parasitemia levels. The PCR detected more three positives in one CSO, one Japanese sparrowhawk (*Accipiter gularis*), and one OSO. The sensitivity and specificity of the PCR method were 93.3% and 99.1%, respectively. The overall infection rate was very low (4.9%). The highest infection rate was recorded in cold-dry season (9.9%). Coinfection of *Plasmodium* with trypanosomes was found in all three ABOs. Coinfection with *Haemoproteus* spp. was found in one BO, three CSOs, and one SO. Coinfection with *Haemoproteus* spp. and *Leucocytozoon danilewskyi* was found in the OSO. Microfilarias were detected in one ABO and one CSO. The ultrastructure of trypomastigotes of *T.* cf. *corvi* in an ABO revealed fine structures. All small subunit ribosomal RNA (SSU rRNA) sequences belong to two clades: *T. avium* and *T. corvi-culicavium* complex/group. SSU rRNA gene amplification was not successful in one BO. The raptors with trypanosome infections showed normal hematological values and healthy appearance. Furthermore, this is the first report of *T. avium* in a nocturnal raptor from Thailand.

Keywords Avian · Haemosporidian · Infection rate · rRNA · Ultrastructure

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Introduction

Avian trypanosomes (Kinetoplasta, Trypanosomatidae) are transmitted by blood-sucking insects, such as mosquitoes and biting flies (Votýpka et al. 2012), by ingestion or conjunctiva contamination (Votýpka et al. 2004; Svobodová et al. 2017). Avian trypanosomes are in most cases harmless to their hosts (Salakij et al. 2012a; Peirce 2016). Due to parasitemia, however, there was a report of some clinical signs in *T. avium*-infected falcons, including lethargy, weakness, inability to fly high, weight loss, dyspnea, and death (Tarello 2005). There have been some reports of trypanosomes in raptors, including *T. avium* Danilewskyi, 1885; *T. bennetti* Kirkpatrick et al., 1986; *T. corvi* Stephens and Christopher, 1908; and *T. noctuae* Schaudinn, 1904, in little owls, *Athena noctuae*

(Votýpka et al. 2012), and unidentified species (Leppert et al. 2008; Svobodová et al. 2015). Recently, in Thailand, there was a report of a *T. corvi* infection in a shikra, *Accipiter badius* Gmelin, 1788 (Salakij et al. 2012a).

Avian trypanosomes have been described in approximately 100 species based on morphological characteristics and host-specific factors, but only a few morphological features have been sufficiently described because of poor illustrations and descriptions (Valkiūnas et al. 2011). Moreover, there is evidence that avian trypanosomes are not host specific (Bennett 1961, 1970b). Votýpka and Svobodová (2004) were successful for experimental transmission of *T. avium*, isolated from naturally infected common buzzards (*Buteo buteo* Linnaeus, 1758) to canaries (*Serinus canaria*) via black flies (*Eusimulium latipes*). Although parasite morphometry may vary during maturation, their morphological characteristics (body shape, free flagellum, undulating membrane, and the position and morphology of the kinetoplast) are conserved (Valkiūnas et al. 2011)

Avian haemosporidians (Haemosporidae) of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* are widespread blood parasites in bird species worldwide (Valkiūnas et al. 2011; Peirce 2016). Although avian trypanosomes and avian haemosporidians are phylogenetically unrelated, the life cycles and transmission modes of their vector species share several similarities (Valkiūnas 2005; Hanel et al. 2016). Coinfection with haemosporidians and trypanosomes has been extensively documented (Leppert et al. 2008; Svobodová et al. 2015; Peirce 2016; Soares et al. 2016). A report of a coinfected with *T. corvi* and *Plasmodium circumflexum* was documented in a shikra from Thailand (Salakij et al. 2012a, b).

A phylogenetic study, using a fragment of the small subunit ribosomal RNA (SSU rRNA) gene of the avian trypanosome, was applied to describe the genotype of the trypanosome and the relationship between the avian host and the collection site (Sehgal et al. 2001). It was also used to describe the relationships among avian trypanosomes (Votýpka et al. 2002; Votýpka and Svobodová 2004; Svobodová et al. 2017). Phylogenetic analysis of the SSU rRNA genes and the random amplication of polymorphic DNA (RAPD) divided the trypanosome isolates from birds and bloodsucking Diptera into 11 separate lineages (Zidková et al. 2012).

In Thailand, raptors are legally protected. Their health is tracked and treated by the Kasetsart University Raptor Rehabilitation Unit (KURRU). Infected raptors, raptors with other health concerned (trauma or malnutrition), and orphaned raptors are admitted to this unit (Kidsin et al. 2012). Blood parasites, mostly *Haemoproteus* spp., have been found in raptors admitted to the KURRU (Pornpanom et al. 2014; Salakij et al. 2012b, 2015, 2018). Reports of trypanosome infections in raptors from Thailand are scarce (Salakij et al. 2012a), although the habitats allow the birds to come into contact with insect vectors. Thus, the aims of this study were to detect trypanosome infections in raptors admitted to the KURRU from Thailand, including hematology, morphology, morphometry, ultrastructure, infection rate, and the phylogeny of the trypanosomes.

Materials and methods

Study site and sample collection

All 364 raptors (214 adults (58.8%), 147 juvenile (40.4%), and 3 nestling (0.8%)) were opportunistically admitted to KURRU, Faculty of Veterinary Medicine, Kasetsart University, Khamphaeng Sean, Thailand (14°1′N, 99°58′E), during September 2012 to May 2018. They were birds from 22 species of diurnal raptors and 12 species of nocturnal raptors. Barn owls (BO, *Tyto alba* Scopoli, 1769, $n = 65$), black-shouldered kites (BSK, *Elanus caeruleus* Desfontainers, 1789, $n = 49$), Brahminy kites (BK, *Haliastur indus* Boddaert, 1783, $n = 50$), and collared scops owls (CSO, *Otus lettia* Hodgson, 1836, $n = 38$) were the most commonly admitted raptors. Within 14 days after admission, blood samples were collected from all raptors during the morning. One milliliter of blood was collected from the jugular vein of each raptor and transferred to tubes containing ethylene diamine tetra acetic acid (EDTA, Campbell 1995). Immediately, the blood samples were placed in a cooler and transferred to laboratory located approximately 300 m from the KURRU.

Hematology

Blood smears were prepared within 30 min after the blood collection, air-dried, and stained with Wright's stain [an in-house preparation using Wright's eosin methylene blue and Giemsa's azure eosin methylene blue (Merck KGaA, Darmstadt, Germany)] for the grading of blood parasite infections, morphologic evaluations of red blood cells (RBCs) and white blood cell (WBC) differentiation.

Complete blood cell counts were obtained using previously described methods (Campbell 1995; Salakij et al. 2012b, 2015). Briefly, packed cell volumes (PCVs) were determined using a microhematocrit centrifuge at 10,000×*g* for 5 min. Total RBC and WBC counts were determined manually using a counting chamber after the blood had been diluted 200 times with Natt and Herrick's solution, as described previously (Campbell 1995; Salakij et al. 2015). A corrected WBC count was calculated by applying the following formula: WBC × 100 / (100 + thrombocyte percentage). The hemoglobin (Hb) concentration was determined by the cyanmethemoglobin method, in which free RBC nuclei were removed by centrifugation before reading the absorbance. Reticulocyte counts (aggregate and punctate) were determined by staining with new

methylene blue with a wet preparation. Total protein and fibrinogen concentrations were determined by using a refractometer (Atago Ltd., Tokyo, Japan) and the heat precipitation method for fibrinogen. WBC differential counts were based on an average count of 200 cells by two veterinary hematologists. Thrombocytes per 100 WBCs (expressed as the thrombocyte percentage) were also counted.

Light microscopy examination

All birds were screened for trypanosomes and haemosporidians by light microscopic examination (LM) of two blood smears from each sample by two experienced hematologists. Trypanosome parasite screening was performed in low power field (LPF, × 100 magnification) for 10 min per slide, average 100 fields. Then, the number of trypomastigotes were graded from 10 LPFs of each positive sample (Salakij et al. 2012a). The actual counting of the number of haemosporidian parasites per 10,000 red blood cells was performed at oil-immersion magnification (× 1000 magnification) to estimate the level of infection (Valkiūnas 2005). Species identification based on morphological characteristics was performed under an oil-immersion lens, following the methods outlined in previous reports (Bennett 1970a; Nandi and Bennett 1994; Votýpka and Svobodová 2004; Salakij et al. 2012a).

Morphology and morphometry

Pictures of trypomastigotes were prepared from each positive blood smear using a BX53 light microscope equipped with a DP73 digital camera and cellSens Standard imaging software (Olympus, Tokyo, Japan). Some morphometric parameters of trypomastigotes were obtained from previous reports (Valkiūnas et al. 2011; Svobodová et al. 2017). According to Nandi and Bennett (1994) which divided *T. corvi* into the following three forms according to the width of the body through center of nucleus (BW): slender (3.5–4.5 μm), intermediate (4.5–7.0 μm), and broad (> 7.0 μm). A BW less than 3.5 μm was classified as developing. Therefore, this report includes four forms of *T. corvi.* The species of coinfected *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon danilewskyi* (avian haemosporidians) were detected by their morphology (Valkiūnas 2005).

Statistical analysis

For statistical analysis, the normal distribution of morphometric data was checked by the function "shapiro.test" (Shapiro-Wilk test). The function "kruskal.test" (Kruskal-Wallis test) and "kruskalmc" (multiple comparison test) in the "pgirmess" package was applied to identify significant differences of morphometrics among groups for each parameter. All functions and packages are available in R language (R development core team 2016).

Trypanosome infection rate

Infection rates (%) were calculated from combined data for each year; however, in 2012 and 2018, the infection data did not represent a whole year. Samples were collected from September, 2012 to May, 2018. The 95% confidence intervals (CIs) were calculated by the function bionom.approx in the epitools package (R development core team 2016). Infection rates were classified as seasonal rate following the Meteorological Department of Thailand, including three seasons; cold-dry (late October to early February), summer (late February to early May), and rainy (late May to early October).

Ultrastructure of *Trypanosoma corvi*

EDTA-anticoagulated samples from one Asian barred owlet (ABO, *Glaucidium cuculoides* Vigors, 1831, KU220) were processed for transmission electron microscopy (TEM) as described previously (Salakij et al. 2012a). Briefly, the buffy coats from microcapillary tubes were fixed immediately after micro-centrifugation in 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 24 h, post-fixed in 1% osmium tetroxide for 2 h, and embedded in Spurr's epoxy resin. The fixation of the buffy coats was performed within 30 min after blood collection. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEM 1230 TEM at 80 kV (JEOL Ltd., Tokyo, Japan).

Molecular detections

All birds were screened for trypanosomes by nested polymerase chain reactions (PCRs). Total DNA was extracted from 50 μl of each blood sample, using Blood Genomic DNA Extraction kits (FavorPrep™, Favorgen Biotech Corp., Taiwan). The SSU rRNA gene fragment was amplified by the nested PCR (Valkiūnas et al. 2011). For the primary reaction, 2 μl of each extracted DNA sample in DreamTaq Green PCR Master Mix (2×) (ThermoScientific™, USA) and 10 nM primers (Tryp763 and Tryp1016) were used. The primary products were diluted with 10-fold RNase-free water, and 2 μl aliquots of those solutions were used as secondary templates. The primers for the secondary templates were Tryp99 and Tryp957. Amplicons (770 base pairs; bp) of the SSU rRNA gene were stained with fluorescent dye (GelRed® nucleic acid gel stain, Biotium Inc., CA, USA) and electrophoresed on a 1.5% agarose gel at 132 V for 20 min. There were no template controls and positive controls in any of the reactions used to avoid false reactions.

The genomic DNA from each blood sample was analyzed by nested PCR to amplify a fragment of cytochrome *b* (cyt *b*)

for avian haemosporidian mitochondrial DNA and were performed as previous report (Hellgren et al. 2004; Cosgrove et al. 2006). Specific primers designated as HaemNF and HaemNR2 were used for the primary PCR amplification using recombinant *Taq* DNA polymerase (Invitrogen, Waltham, Massachusetts, USA).

DNA sequencing and phylogeny

The amplicons of the SSU rRNA gene were extracted using GEL/PCR Purification Mini Kit (FavorPrep™, Favorgen Biotech Corp., Taiwan). Purified DNAs were submitted to Apical Scientific (Selangor, Malaysia) for directional nucleotide sequencing. The forward and reverse strands of each sequence were aligned and contiged by using BioEdit version 7.2.5 (Hall 1999).

The new SSU rRNA isolates were deposited in GenBank, with the accession numbers MH549533-MH549546 and MK516190-MK516192 (Table 2). The tree topology was generated using 64 sequences of SSU rRNA gene of avian trypanosomes. Seventeen sequences were our reported sequences from raptors in KURRU, Thailand. Forty-seven sequences were from previous reported (Zídková et al. 2012), including 35 lineage sequences isolated from avian host and 12 sequences were isolated from insects. Two sequences of amphibian trypanosomes were used as the out groups, including *Trypanosoma rotatorium* (B2-II, AJ009161) and *T. mega* (ATCC30038, AJ009157). The consensus length was 799 base pairs (bp). MEGA 7 software (Kumar et al. 2016) was used to generate the maximum-likelihood phylogeny with 1,000 bootstrap replicates. The calculation of the distance values and finding the best-fit model of evolution were performed using the Kimura two-parameter model with grammar distribution (Kimura 1980).

Results

Under light microscope, avian trypanosomes were found in 15 raptors (Table 1) including one BSK (Fig. 1a), one Brahminy kite (BrK, *Haliastur indus*, Fig. 1b), two mountain hawk-eagles (MHEs, *Spizaetus nipalensis* Hodgson, 1836, Fig. 1c), three ABOs (Figs. 1d–f), three BOs (Fig. 1g, h), three CSOs (Fig. 1i, j), one spotted owlet (SO, *Athene brama* Temminck, 1821, Fig. 1k), and one oriental scops owl (OSO, *Otus sunia* Hodgson, 1836, Fig. 1l). The number of trypomastigotes in the peripheral blood of all infected raptors was very low (less than 1 trypomastigote per LPFs). There were no clinical signs in any raptor even when they were infected with trypanosomes. The overall trypanosome infection rate in raptors including both LM and PCR was 4.9%. The infection rate in diurnal raptors (2.6%) was lower than that in nocturnal raptors (7.6%, Table 1).

Table 1 *Trypanosoma* (*T.*) spp. positive raptors by light microscopy (LM) and nested polymerase chain reactions (PCR)

Raptors	LM	Nested PCR	Infection rate
Diurnal raptors ($n = 5$)		Total	5/193 (2.6%)
Black-shouldered kite	1 (*T.* cf. *corvi*)	1	1/49 (2.0%)
Brahminy kite	1 (*T.* cf. *corvi*)	1	2/50 (4.0%)
Japanese sparrow hawk	1[a] (*T.* sp.)	1[a]	1/1 (100%)
Mountain hawk-eagles	2 (*T.* cf. *corvi*)	2	2/3 (66.7%)
Nocturnal raptors ($n = 13$)		Total	13/171 (7.6%)
Asian barred owlets	3 (*T.* cf. *corvi*)	3	3/27 (11.1%)
Barn owls	3 (*T.* cf. *corvi*)	2	3/65 (4.6%)
Collared scops owls	3 (*T.* cf. *corvi*)	4[b]	4/38 (10.5%)
Spotted owlet	1 (*T.* cf. *corvi*)	1	1/27 (3.7%)
Oriental scops owls	1 (*T. avium*)	2[b]	2/2 (100.0%)
Total	16	17	18/364 (4.9%)

[a] After PCR positive, light microscopic re-examination was positive with only one trypomastigote found under whole blood smear scanning

[b] Light microscopic re-examinations were negative after PCR positive

Four diurnal raptors were infected with only trypanosomes while 11 nocturnal raptors were coinfected with other blood parasites. Coinfection with *Plasmodium* spp. was found in all three ABOs (Supplementary Fig. 1a). Coinfection with *Haemoproteus* spp. was found in one BO, three CSOs (Supplementary Fig. 1b), and one SO (Table 2). Coinfections with *Haemoproteus* spp. (Figs. 1l and Supplementary Fig. 1c) and *Leucocytozoon danilewskyi*, Ziemann, 1898 (Supplementary Fig. 1c) were found in only one OSO (Table 2). The microfilaria were detected in one ABO (Supplementary Fig. 1d), and one CSO (Supplementary Fig. 1e). Microfilaria in these two owls had morphological differences; those found in the ABO contained sheaths and were shorter than those found in the CSO.

Based on morphology, these 15 avian trypanosomes were identified as *T.* cf. *corvi* and *T. avium.* Fourteen cases of infection with *T.* cf. *corvi* were found in four diurnal raptors (Fig. 1a–c), and 10 nocturnal raptors (3 ABOs, Fig. 1d–f; 3 BOs, Fig. 1g, h; 3 CSOs, Fig. 1i, j, l SO, Fig. 1k). The nucleus was round in shape, stained pink, and 17.0 ± 0.5 μm^2 in area. The kinetoplast was small, round, and close to an oval vacuole. The anterior and posterior ends were tapered to points. One *T. avium* with blade-shaped trypomastigotes was found in OSO-KU489 (Fig. 1l). The kinetoplast was small and oval in shape and adhered to oval vacuoles. Both the anterior and posterior ends tapered to points, but the posterior end was narrower than the anterior end (Fig. 1l). The granulated round pink nucleus was close to the undulating membrane.

Supplementary Table 1 shows the hematological values in all the trypanosome-infected raptors. Only *T. corvi*-infected birds showed no changes in the erythrocyte parameters while birds coinfected with avian malaria (*Plasmodium* spp. and

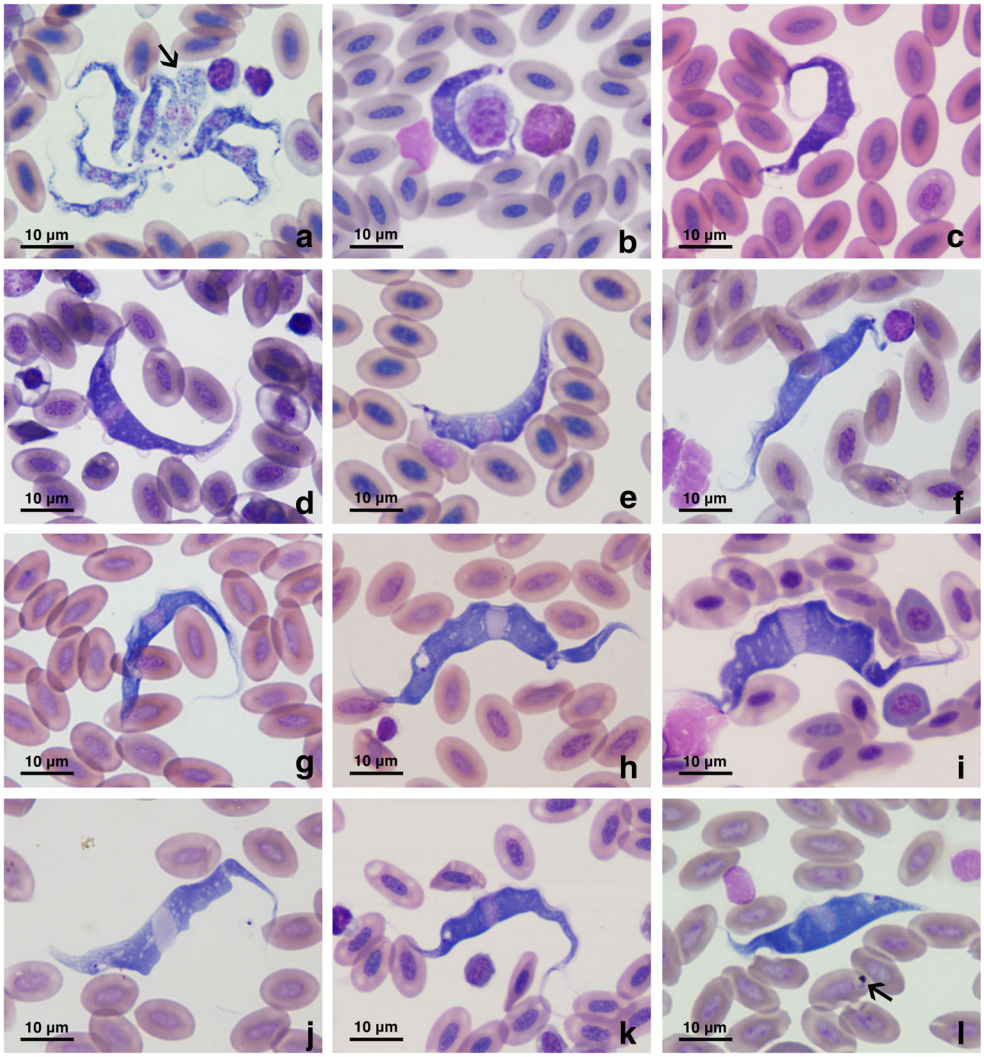

Fig. 1 Trypomastigotes of trypanosomes in raptors. *Trypanosoma* cf. *corvi* in **a** black-shouldered kite (KU484). Clustered trypomastigotes showing binary fission (arrow), **b** Brahminy kite (KU466), **c** mountain hawk-eagle (KU123), **d**–**f** Asian barred owlets (KU213, KU220 and KU454), **g**, **h** barn owls (KU202 and KU327), **i**, **j** collared scops owls (KU127 and KU222), and **k** spotted owlet (KU382). **l** *T. avium* in an oriental scops owl (KU489), coinfection with *Haemoproteus* spp. (arrow). **a** Developing forms. b Slender forms. c–g and k Intermediate forms. h–j Broad forms. Wright's stain

Haemoproteus spp.) had lower erythrocyte values. However, the OSO had a high PCV, even though it was coinfected with *Haemoproteus* spp. and *L. danilewskyi*.

The ultrastructure of *T.* cf. *corvi* in ABO-KU220 revealed trypomastigotes packed between RBCs (Fig. 2) that contained numerous ribosomes, a vesicular nucleus with centered large nucleolus (Fig. 2b), typical axonemes in the flagellum (Fig. 2a, c, d), mitochondria (Fig. 2b), and residual bodies (Fig. 2c). Beneath the plasma membrane, there were cortical or pellicular microtubules. The black/white, round organelles known as acidocalcisomes and homogeneous electron-dense glycosomes were scattered throughout the trypomastigotes (Fig. 2a, c, d). The description of each fine structure of trypomasigotes of *T.* cf. *corvi* in our study was followed Stephen (1986).

The overall infection rate of trypanosome infected raptors was 4.9%, with a 95% confidence interval (CI) of 2.7 to 7.1. The seasonal infection rates are shown in supplementary Fig. 2. The highest infection rate was recorded in cold-dry season (9.9% and 95% CI 4.0 to 15.7), and the second highest was recorded in summer (5.0% and 95% CI 1.1 to 9.0).

Almost all SSU rRNA genes of trypanosomes in our LM positive samples were amplified and sequenced except one sample from BO-KU202. The PCR detected more three positive samples from LM-negative samples; including one CSO, one Japanese sparrowhawk (JSH, *Accipiter gularis*), and one OSO (Tables 1 and 2). Light microscopic re-examination was positive with only one trypomastigote found under whole blood smear scanning of the JSH while the other two owls were negative. So, the sensitivity and specificity of these primers were 93.3% and 99.1%, respectively. The CSO was coinfected with *Haemoproteus* spp. (MK411009, Table 2). The coinfections of *Haemoproteus* sp. (less than 01% RBC) and *Leucocytozoon* sp. (only one round form microgametocyte, MK650103) were detected in only in the OSO (Table 1).

The phylogenetic trees (Fig. 3) revealed that all 17 SSU rRNA genes were grouped into two major groups. Thirteen *Trypanosoma* cf. *corvi* isolates and *Trypanosoma* sp. CSO-

Table 2 Isolates of 17 *Trypanosoma* spp., raptor data, GenBank accession numbers and coinfections

Isolate	Host species (scientific name)	Host species (English typical name)	Year	Locality	GenBank acc. no.	Coinfections (GenBank acc. no.)
ABO-KU213	*Glaucidium cuculoides*	Asian barred owlet/adult	2014	Nakhon Ratchasi-ma	MH549537	*Plasmodium* sp. (MK390824) + microfilaria
ABO-KU220	*Glaucidium cuculoides*	Asian barred owlet/adult	2014	NA[c]	MH549538	*Plasmodium* sp. (MK390837)
ABO-KU454	*Glaucidium cuculoides*	Asian barred owlet/juvenile	2016	Prachin Buri	MH549539	*Plasmodium* sp. (MK390835)
BO-KU200	*Tyto alba*	Barn owl/adult	2013	Ayutthaya	MH549540	*Haemoproteus* sp. (KU528639)
BO-KU327	*Tyto alba*	Barn owl/nestling	2014	Nakhon Ratchasi-ma	MH549541	None
BSK-KU484	*Elanus caeruleus*	Black-shouldered kite/adult	2016	Ang Thong	MH549536	None
BrK-KU466	*Haliastur indus*	Brahminy kite/adult	2016	NA[c]	MH549535	None
CSO-KU127	*Otus lettia*	Collared scops owl/adult	2013	Rayong	MH549542	*Haemoproteus* sp. (KJ561657)
CSO-KU222	*Otus lettia*	Collared scops owl/adult	2013	Bangkok	MH549543	*Haemoproteus* sp. (KU528643) + microfilaria
CSO-KU621	*Otus lettia*	Collared scops owl/adult	2018	Chaiyaphum	MH549544	*Haemoproteus* sp. (MK411009)
MHE-KU123	*Spizaetus nipalensis*	Mountain hawk-eagle/juvenile	2013	Kanchana Buri	MH549533	None
MHE-KU188	*Spizaetus nipalensis*	Mountain hawk-eagle/adult	2013	NA[c]	MH549534	None
SO-KU382	*Athene brama*	Spotted owlet/adult	2014	Bangkok	MH549545	*Haemoproteus* sp. (MK390802)
OSO-KU489	*Otus sunia*	Oriental scops owl/adult	2015	Nakhon Nayok	MH549546	*Haemoproteus* sp. (MK390807) + *Leocucytozoon danilewskyi* (MF370338)
OSO-KU249[a]	*Otus sunia*	Oriental scops owl/adult	2014	Bangkok	MK516190	*Haemoproteus* sp. + *Leocucytozoon* sp. (MK650103)
CSO-KU482[a]	*Otus lettia*	Collared scops owl/adult	2016	Pathum Thani	MK516192	None
JSH-KU309[b]	*Accipiter gularis*	Japanese sparrowhawk/adult	2016	Ang Thong	MK516191	None

ABO Asian barred owlet, *BO* barn owl, *CSO* collared scops owl, *SO* spotted owlet, *BrK* Brahminy kite, *BSK* black-shouldered kite, *OSO* oriental scops owl, *MHE* mountain hawk-eagle, *JSH* Japanese sparrow hawk

[a] Light microscopic re-examinations were negative after PCR positive

[b] After PCR positive, light microscopic re-examination was positive with only one trypomastigote found under whole blood smear scanning

[c] NA captive, original site not known

KU482 (*T.* sp. in the phylogeny) were close together, with 0.0–0.7% genetic distances. This group was close to lineages I, IV, V, and XII which was previously described (Zídková et al. 2012), with genetic distances 0.1–0.5%, 0.5–0.9%, 0.5–0.9%, and 0.1–0.9%, respectively. *T. culicavium* were grouped as distinct clade which was split from *T. corvi*. These 14 *T.* spp. isolates belonged to *T. corvi-culicavium* complex/group.

The *T. avium* in the OSO was identical to *T. avium* isolated from adult kestrel, *Falco tinnunculus* in the Czech Republic (AY099319), nestling lesser spotted eagle, *Aquila pomarina* in the Slovak Republic (AF416559), and nestling buzzard, *Buteo buteo* (AY099320) in the Czech Republic (Votýpka et al. 2002). *T. avium* OSO-KU489, *Trypanosoma* sp. JSH-KU309, and *Trypanosoma* sp. OSO-KU489 were grouped together and in distinct clade with other six *T. avium* isolates (AF416559, AF416563, AY099319, AY099320, FJ649483, and U39578). These three sequences were grouped in the lineage X + XI of previously described (Zídková et al. 2012) with 0.0–0.1% genetic distances. There are four described species of avian trypanosomes in this phylogeny, including *T. avium*, *T. bennetti*, *T. corvi*, and *T. culicavium*. *T. corvi* were divided into more than one clade.

According to measurements by Nandi and Bennett (1994), supplementary Table 2 shows the mean measurements of the trypomastigotes of *T.* cf. *corvi*. The developing form was found in only one raptor (KU484-BSK, Fig. 1a), the slender form was found in three raptors (KU213-

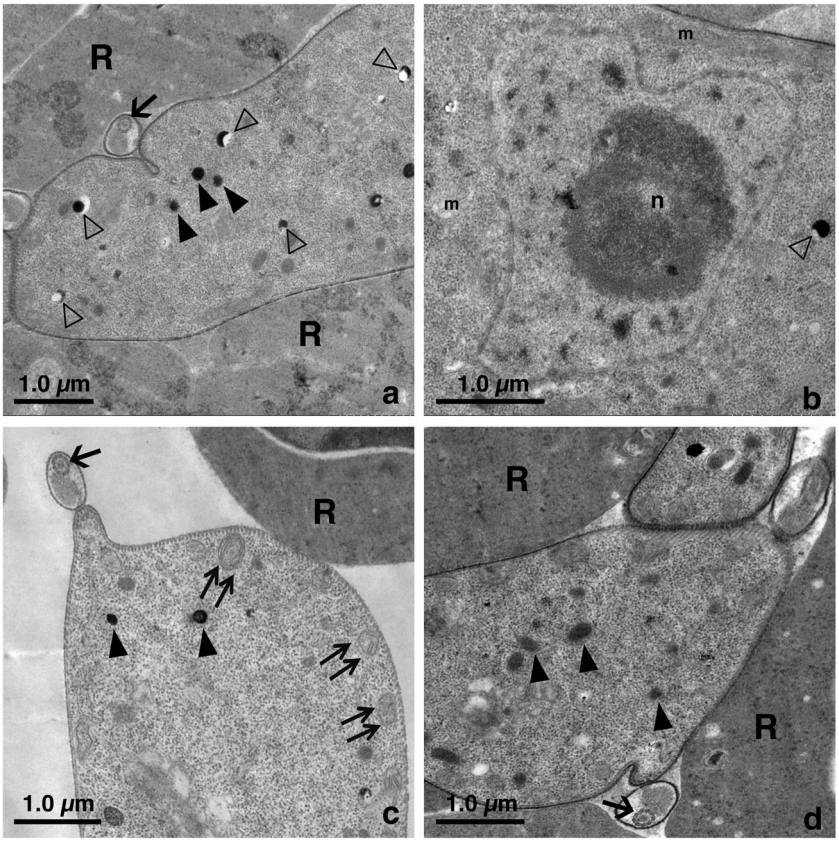

Fig. 2 Ultrastructure of *Trypanosoma* cf. *corvi* in an Asian barred owlet (KU220). Trypomastigotes between RBCs (R) showing typical axonemes in the flagellum (arrows in panels **a**, **c, d**), mitochondria (m in panel **b**), nucleus with a large nucleolus (n in panel **b**), acidocalcisomes (open arrowheads), glycosomes (black arrowheads) and residual bodies (double arrows). Uranyl acetate and lead citrate stains

ABO, Fig. 1d; KU200-BO, KU222-CSO), the intermediate form was found in eight raptors, and the broad form was found in only one raptor (KU327-BO, Fig. 1h). In addition, two or three forms of trypomastigotes were found in the same bird. The average area of the fully grown trypomastigotes (AT, except KU484-BSK) was 136.1 ± 3.9 μm^2 (Supplementary Table 2). The mean length of free flagellum of *T.* cf. *corvi* (11.0 ± 0.3 μm) was longer than that of *T. avium* (8.9 ± 0.6 μm). *T. avium* were larger than *T.* cf. *corvi* with 149.4 ± 5.6 μm^2 of AT (Supplementary Table 2).

The comparative morphometry of the trypomastigotes between different forms of *T.* cf. *corvi* and *T. avium* is shown in Table 3. We excluded the morphometry of the trypomastigotes of KU484-BSK because they were developing trypomastigotes. In addition, there was a group of small trypanosomes that were dividing (Fig. 1a). Three forms of *T.* cf. *corvi* were significantly narrower and longer than *T. avium*, with a longer total length of the trypomastigotes without the free flagellum (PA). In agreement with the longer PA of *T.* cf. *corvi*, the lengths from the center of the nucleus to the anterior end (NA), from the posterior end to the center of the nucleus (PN), and from the kinetoplast to the center of nucleus (KN) were also significantly longer than those of *T. avium* (Table 3). The areas of the kinetoplast (AK) in three forms of *T.* cf. *corvi* were smaller than that of *T. avium* (0.8 ± 0.0 μm^2).

Discussion

Trypanosoma cf. *corvi* was the most common trypanosome identified over the course of 6 years of observations; it was found in 14 of the 15 light microscopic positive raptors in Thailand which were both diurnal and nocturnal. Hence contrast to the findings in Australia, *T. avium* was the most common trypanosome found in birds (Cooper et al. 2017). Nevertheless, this is the first report of a *T. avium*-infected nocturnal raptor in Thailand. The infection rate of trypanosomes in raptors from Thailand was low in comparison to the rates found in other studies in northern goshawks from the Czech Republic (Hanel et al. 2016) and in common buzzards (*Buteo buteo*) and Eurasian sparrow hawks (*Accipiter nisus* Linnaeus, 1758) from the Czech Republic (Svobodová et al. 2015). These differences might be due to different geographic distributions or random population in the KURRU, although raptor habitats are open lands where it is easy to come into contact with insect vectors.

Prolonged exposure to EDTA may cause cellular degeneration in the blood of some species of birds (Campbell 1995); therefore, blood smears were made within 30 min or as soon

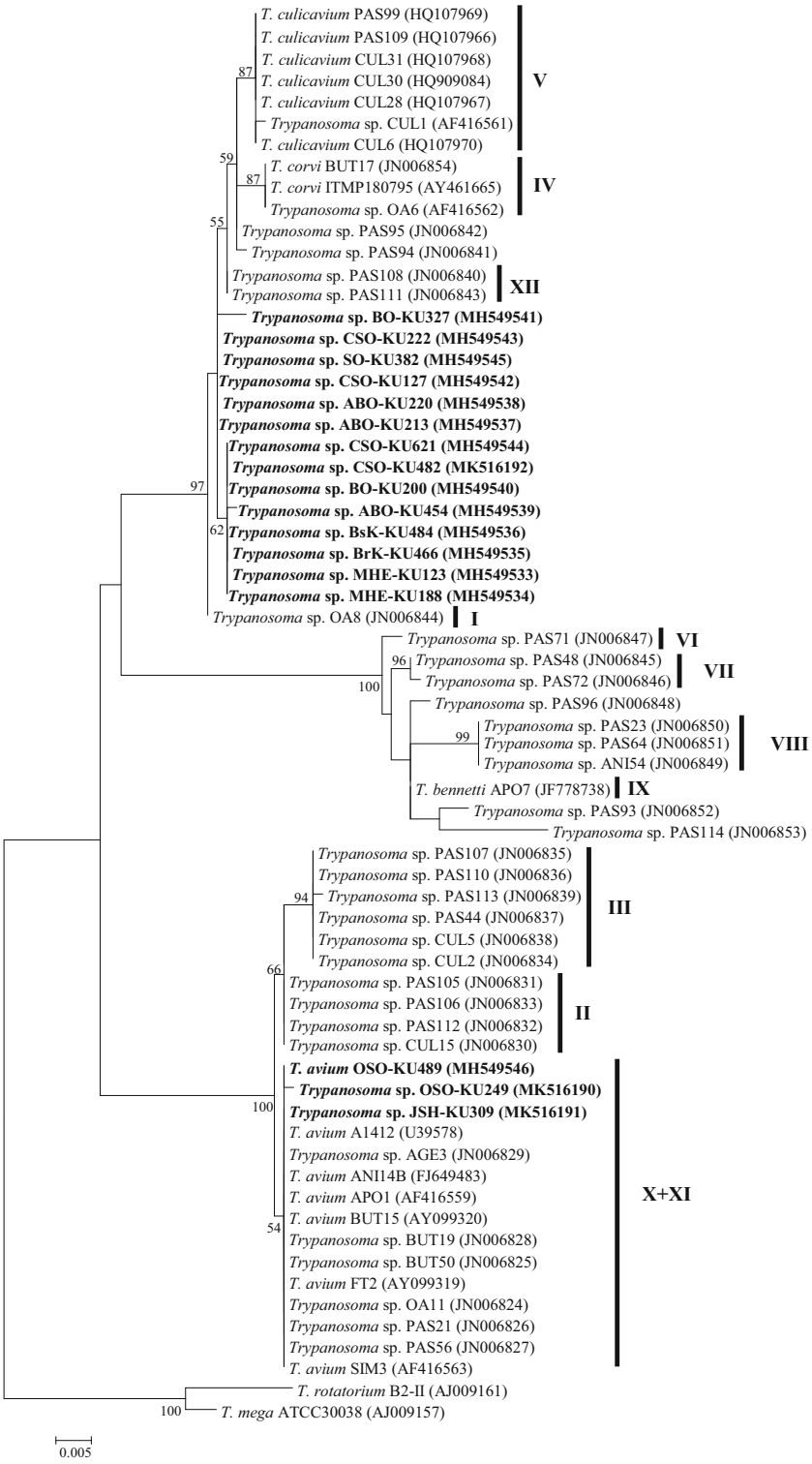

Fig. 3 Phylogenetic trees based on small subunit ribosomal RNA (SSU rRNA, 799 bp) of 17 avian trypanosomes isolated (in bolds) from raptors in Thailand. Sixteen *Trypanosoma* spp. were isolated from three Asian barred owlets (ABO), two barn owls (BO), one Brahminy kite (BrK), one black-shouldered kite (BSK), four collared scops owls (CSO), one Japanese sparrowhawk (JSH), two mountain hawk-eagles (MHK), one spotted owlet (SO) and one oriental scops owl (OSO). *T. avium* was isolated from one OSO. Reported lineages in this study are given in bold. Vertical bar indicate group of avian trypanosomes lineages (clade I-XII) following Zídková et al. (2012). Bootstrap values greater than 50% are shown at the branch points

as possible following blood collection. These were in accordant with human malaria diagnosis that the blood smears should be made as soon as possible in the case of using anticoagulant, certainly less than 3 h to prevent parasite and red cell morphology artifacts (Warhurst and Williams 1996). A delay between blood collection and blood film preparation also creates an increase in number of smudge cells on the blood film (Campbell 2015).

The RBC counts of a BSK and a BrK-infected with only trypanosomes were within the normal reference values for birds (Campbell 2015). The normal hematological values in raptors with single trypanosome infections with low levels of

Table 3 Comparative morphometry (median ± SE) between three forms trypomastigotes of *Trypanosoma* cf. *corvi* and *T. avium*

Parameters	*T.* cf. *corvi*[a]			*T. avium*
	Slender form	Intermediate form	Broad form	
Numbers	24	68	24	10
AT (μm^2)	113.9 ± 4.9a	142.0 ± 4.5b	170.0 ± 10.4b	149.4 ± 5.6b
BW (μm)	3.8 ± 0.1a	5.5 ± 0.1b	7.2 ± 0.2c	6.4 ± 0.3bc
PA (μm)	52.4 ± 1.5a	50.7 ± 1.0a	56.3 ± 2.5a	40.5 ± 1.1b
AN (μm^2)	11.5 ± 0.4a	18.1 ± 0.4b	22.5 ± 0.7c	18.8 ± 1.1bc
AK (μm^2)	0.5 ± 0.0a	0.6 ± 0.0a	0.6 ± 0.0ab	0.8 ± 0.0b
FF (μm)	10.4 ± 0.8	11.7 ± 0.4	10.8 ± 0.7	8.9 ± 0.6
NA (μm)	27.2 ± 1.2a	29.2 ± 0.6a	30.3 ± 1.6a	20.8 ± 0.6b
PN (μm)	21.0 ± 0.9ab	20.8 ± 0.7a	24.5 ± 1.5a	17.2 ± 0.6b
KN (μm)	12.6 ± 0.3a	13.1 ± 0.2b	13.6 ± 0.6b	9.7 ± 0.1b
PK (μm)	6.8 ± 0.6	7.3 ± 0.5	9.1 ± 1.0	6.3 ± 0.6
BW/PA	0.1 ± 0.0a[a]	0.1 ± 0.0a	0.1 ± 0.0ab	0.2 ± 0.0b
PK/PA	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
PN/NA	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.9 ± 0.0
PN/PA	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
PN/KN	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.9 ± 0.1

Different lowercase letters indicate significant differences between groups ($p < 0.05$)

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

[a] Exclude trypomastigotes in black-shouldered kite-KU484

parasitemia may indicate that trypanosome infection in raptors has low pathogenicity or accidental findings as previously documented (Salakij et al. 2012a; Campbell 2015; Peirce 2016). The high PCV in an OSO might indicate that *T. avium* had a low pathogenicity in this owl, contrasted with the findings in the study by Tarello (2005), which reported some clinical signs in falcon infected with *T. avium*.

Svobodová et al. (2015) reported the coinfections with *Leucocytozoon* and *Trypanosoma* were more frequent than coinfections with *Leucocytozoon* and *Haemoproteus* in buzzards and sparrow hawks due to their transmission by different vectors. However, our study revealed the opposite results. The *T.* cf. *corvi* infections in the four and five species of diurnal and nocturnal raptors respectively were not species specific, which is consistent with the findings of other studies (Bennett 1961, 1970b; Votýpka and Svobodová 2004; Salakij et al. 2012a; Zídková et al. 2012). Our study also supported the previous finding that trypanosomes are frequently observed in mixed infections with other hematozoa (Peirce 2016). Microfilariae are the larval stages of filarial worms, of which there are many genera and species that occur in birds (Peirce 2016); those found in the ABO and CSO in this study had different morphologies. Microfilariae are considered nonpathogenic and cause little harm to their bird hosts (Campbell 2015).

The morphometric values of *T.* cf. *corvi* and *T. avium* were statistically compared in this study, and the morphology indicated that *T.* cf. *corvi* is narrower and longer than *T. avium*. According to Nandi and Bennett (1994), *T.* cf. *corvi* in raptors are found in the following three forms: slender, intermediate, and broad forms. The appearance of the broad from of the trypomastigotes of the *T.* cf. *corvi* might be confused with those of *T. avium*. However, the other trypomastigote characteristics were distinguishable between these two trypanosome species, such as the nucleus and cytoplasm. *T. corvi* has a granulated nucleus, but that of *T. avium* showed solid pink staining. The cytoplasm of *T. corvi* was vacuolated compared with the striated cytoplasm of *T. avium* (Baker 1956; Nandi and Bennett 1994).

Despite that some morphometrics vary during the development of the same *Trypanosoma* strains even in different avian hosts, the gross morphological characteristics of hematozoic trypomastigotes are relatively stable (Valkiūnas et al. 2011). In particular, fully grown trypomastigotes have conserved morphological characteristics even when coinfected with other pathogen (Valkiūnas et al. 2011; Sehgal et al. 2015). The group of small trypomastigotes was found in BSK-KU484 which seemed to be smaller than the other *T.* cf. *corvi* but with similar morphologies. This difference may be associated with rapid binary fission during development. Blood

trypomastigote morphology still was an important criterion for species determination of avian trypanosomes (Valkiūnas et al. 2011).

The pattern of phylogenetic branches was the same as previous reports (Zídková et al. 2012; Šlapeta et al. 2016). According to Zídková et al. (2012) description, the SSU rRNA phylogeny was congruent with the RAPD analysis. Their SSU rRNA phylogeny described three majority groups of 12 avian trypanosomes lineages. Our phylogenetic revealed the same results as follows: the first group contained lineages I, IV, V, and XII. The second group was contained lineages VI, VII, VIII, and IX. The third group contained lineages II, III, and X + XI. Our 13 *T.* cf. *corvi* and *Trypanosoma* sp. CSO-KU482 were separated from reported lineages. They may be the new lineages. However, the lineage confirmation needs more study such as using RAPD method (Zídková et al. 2012). *T. avium* OSO-KU489, *Trypanosoma* sp. JSH-KU309, and *Trypanosoma* sp. OSO-KU249 were grouped in lineage X + XI clade, which also should be confirmed by the other methods. Although we did not describe the exact lineage of our trypanosomes, this phylogeny provided interesting point for the further study.

Our results revealed polyphyly of avian trypanosomes that were in agreement with previous studies (Votýpka et al. 2004, 2012; Zidková et al. 2012). The phylogeny of the SSU rRNA genes revealed that *T. avium* comprised a monophyletic group even though they were isolated from different raptors. They are also grouped in the same clade as other *T. avium* FT2 (AY099319, European kestrel, *Falco tinnunculus*), *T. avium* APO1 (AF416559, lesser spotted eagle, *Aquila pomarina*), and *T. avium* A1412 (U39578, raven) (Votýpka et al. 2004), in which European kestrel and lesser spotted eagle were diurnal raptors. Our study revealed the first record of *T. avium* in nocturnal raptors, oriental scops owl. This suggests that *T. avium* can be transmitted across various species of avian hosts. Hence, these data also supported the lack of host specificity of avian trypanosomes (Votýpka et al. 2002; Votýpka and Svobodová 2004; Valkiūnas et al. 2011) and increased the list of vertebrate hosts of both *T. avium* and *T.* cf. *corvi*.

In our previous report of *T. corvi* in a shikra (Salakij et al. 2015), we used short primer set (326 bp) for 18S rRNA (Sehgal et al. 2001). But in this study, we use the new primer set (Tryp99 and Tryp957) to amplify SSU rRNA gene, which provides a longer sequence (Valkiūnas et al. 2011) and we found more three PCR positive samples. So, these primers may be useful for analyzing the phylogenetic tree of SSU rRNA of avian trypanosomes.

In summary, our study reported the combined hematologic, microscopic, morphometric, monthly infection rates and molecular information of raptor trypanosome infections in Thailand. These data are considered to be baseline information and can be used to monitor blood parasites in the raptors after admission.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics approval This study was approved by the Institutional Laboratory Animal Care and Use Committee of Kasetsart University, Thailand under protocol number ACKU 01560.

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