Intercontinental distribution of a new trypanosome species from Australian endemic Regent Honeyeater (*Anthochaera phrygia*)

JAN ŠLAPETA[1]*, VICTORIA MORIN-ADELINE[2], PAUL THOMPSON[3], DENISE MCDONELL[2], MICHAEL SHIELS[3], KATRINA GILCHRIST[2], JAN VOTÝPKA[4] *and* LARRY VOGELNEST[3]

[1] *Faculty of Veterinary Science, School of Life and Environmental Sciences, The University of Sydney, New South Wales 2006, Australia*
[2] *Faculty of Veterinary Science, The University of Sydney, New South Wales 2006, Australia*
[3] *Taronga Zoo, Taronga Conservation Society Australia, Bradleys Head Road, Mosman, New South Wales 2088, Australia*
[4] *Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic*

(*Received 25 September 2015; revised 2 February 2016; accepted 5 February 2016; first published online 22 March 2016*)

SUMMARY

Establishing a health screening protocol is fundamental for successful captive breeding and release of wildlife. The aim of this study was to undertake a parasitological survey focusing on the presence of trypanosomes in a cohort of Regent Honeyeaters, *Anthochaera phrygia*, syn. *Xanthomyza phrygia* (Aves: Passeriformes) that are part of the breeding and re-introduction programme carried out in Australia. We describe a new blood parasite, *Trypanosoma thomasbancrofti* sp. n. (Kinetoplastida: Trypanosomatidae) with prevalence of 24·4% (20/81) in a captive population in 2015. The sequence of the small subunit rRNA gene (SSU rDNA) and kinetoplast ultrastructure of *T. thomasbancrofti* sp. n. are the key differentiating characteristics from other *Trypanosoma* spp. *T. thomasbancrofti* sp. n. is distinct from *Trypanosoma* cf. *avium* found in sympatric Noisy Miners (*Manorina melanocephala*). The SSU rDNA comparison suggests an intercontinental distribution of *T. thomasbancrofti* sp. n. and *Culex* mosquitoes as a suspected vector. Currently, no information exists on the effect of *T. thomasbancrofti* sp. n. on its hosts; however, all trypanosome-positive birds remain clinically healthy. This information is useful in establishing baseline health data and screening protocols, particularly prior to release to the wild.

Key words: *Trypanosoma*, conservation, avian host, distribution, reintroduction.

INTRODUCTION

The iconic Regent Honeyeater, *Anthochaera phrygia* (Shaw, 1794) syn. *Xanthomyza phrygia* (Aves: Passeriformes) is a critically endangered passerine endemic to South-Eastern Australia. Historically, this bird could be seen in flocks of hundreds, ranging from Queensland to South Australia (Driskell and Christidis, 2004; Gardner *et al*. 2010; Liu *et al*. 2014). The current estimated wild population of less than 400 birds is distributed across four key sites in Victoria and New South Wales (Franklin *et al*. 1989; Thomas, 2009). Extensive loss of box-ironbark eucalyptus forests is attributed as the primary threatening process for this species throughout its range. The Regent Honeyeater is a highly mobile species that covers extensive distances to feed on sporadically available nectar and insects within box-ironbark eucalypt forests. In an effort to prevent extinction, the species has been the subject of an intensive recovery programme for the past 20 years. In 1995, nine male and nine female founders were collected from Chiltern (Victoria), and Capertee Valley (New South Wales) in a captive trial to provide some level of insurance from extinction. A small captive breed-and-release strategy began in 2000 and implemented into the programme in 2008. From the original founders, 312 chicks had been produced by 2013 and of these, 117 have been released (Liu *et al*. 2014).

Identification and knowledge of the life history of infectious agents in wildlife is imperative for the implementation of satisfactory recovery programs. In a previous parasitological survey of a cohort of the Regent Honeyeaters at Taronga Zoo, Australia, we described a coccidian (*Isospora lesouefi*) present in essentially all captive birds, displaying diurnal periodicity of oocyst shedding (Morin-Adeline *et al*. 2011). The ecological significance of an ongoing *I. lesouefi* infection in the captive and the wild birds is unknown, nevertheless, recent observations on released birds showed that they thrive and

* Corresponding author. Faculty of Veterinary Science, School of Life and Environmental Sciences, The University of Sydney, McMaster Building B14, New South Wales 2006, Australia. E-mail: jan.slapeta@sydney.edu.au

doi:10.1017/S0031182016000329

pair with wild birds (Dean Ingwersen, personal observations). Besides coccidian and other particularly monoxenous intestinal parasitic organisms, birds are host to several groups of dixenous blood parasites including protists such as haemosporidia (Haemospororida) and trypanosomes (Kinetoplastida, Trypanosomatidae) (Baker, 1976; Valkiūnas, 2005).

In contrast to their mammalian relatives, avian trypanosomes are in most cases harmless to their hosts and remain understudied. Avian trypanosomes rarely cause clinical disease in domestic birds (Baker, 1976), while the impact of *Trypanosoma* spp. on wild birds are scarcely documented (Molyneux and Gordon, 1975; Molyneux *et al*. 1983). The increasing avian trypanosome diversity, however, along with new species descriptions now attracts more attention on this rather neglected group of avian parasites (Votýpka *et al*. 2002; Votýpka and Svobodová, 2004; Zídková *et al*. 2012). The majority of known trypanosome species infect mammals, and only about one-fifth are described from birds (Podlipaev, 1990; Sehgal *et al*. 2006). Presumed host specificity has been used as a criterion for species designation, although host–parasite relationships among avian trypanosomes are poorly understood (Zídková *et al*. 2012). One bird species can harbour several trypanosome species (Molyneux and Gordon, 1975; Votýpka and Svobodová, 2004; Zídková *et al*. 2012). Such findings support the hypothesis of a high trypanosome specificity towards its vector (Apanius, 1991; Votýpka *et al*. 2012). Various blood-sucking insect groups such as flat flies (hippoboscids), black flies (simuliids) and mosquitoes are suggested as possible vectors, yet transmission of the majority of avian trypanosomes still remains unclear. Local transmission and distribution is suggested for many avian trypanosome lineages, while on the other hand, some species defined by gene sequence data are probably widespread and distributed intercontinentally (Sehgal *et al*. 2001; Votýpka and Svobodová, 2004; Zídková *et al*. 2012).

The aim of this study was to undertake a parasitological survey focusing on the presence of trypanosomes in a cohort of Regent Honeyeaters that were part of a breeding and reintroduction programme at Taronga Zoo, Australia. We describe a novel trypanosome species, *Trypanosoma thomasbancrofti* sp. n., parasitizing an Australian passerine bird. The SSU rDNA of this new *Trypanosoma* species from the Regent Honeyeater is almost identical (>99·5%) to trypanosomes of the Common Chiffchaff (*Phylloscopus collybita*) and the common house mosquito (*Culex pipiens* complex) from Central Europe. The results suggest an intercontinental distribution of the newly described trypanosome and *Culex* mosquitoes as a suspected vector. This information is useful in establishing appropriate health screening protocols for the Regent Honeyeater, particularly prior to release into the wild.

MATERIAL AND METHODS

Blood collection, blood smears and cultivation of trypanosomes

Blood samples were collected from Regent Honeyeaters as part of the pre-release health screening protocol at Taronga Zoo, Mosman, Australia in 2013–2015. In June 2015, blood samples were also collected from one wild bird, one 2013 released captive bred bird and from 16 of the 2015 captive bred birds that were released in April 2015 when they were recaptured for refitting of transmitters as part of ongoing post-release monitoring in Chiltern, Victoria, Australia. Blood samples were collected post-mortem from the hearts of wild Noisy Miners (*Manorina melanocephala*), trapped and euthanased at Taronga Zoo, Mosman, Australia in May 2013. Noisy Miners are considered a pest bird species that lead to disruptive consequences to other birds (MacNally *et al*. 2012). Periodic reduction of Noisy Miner numbers is undertaken under license MWL000100542 issued by the NSW Government Office of Environment and Heritage (NSW Parks and Wildlife Service).

Blood (10 μL) was taken directly from the birds and used for standard blood smear, methanol fixed and stained with Diff-Quick stain. All blood smears were inspected (5 min each) by a single observer (PT) using an upright microscope at 40× and 100× objective under immersion oil for the presence of trypanosomes and other blood parasites. Blood (50 μL) was taken directly from the birds and placed immediately into a culture flask. Samples were cultured *in vitro* in 15 mL glass cultivation flasks containing a biphasic medium (SNB-9) consisting of a sloping sheep blood agar with premixed vitamins (58980C, Sigma, Australia), then overlaid with a liquid phase supplemented with neomycin (80 μg mL^{-1}). Cultivation was performed at 25–30 °C in a horizontal position with 10° slope. The flasks were checked every 2–6 days for the presence of trypanosomes. From positive samples, an aliquot (200 μL) was taken, and then pelleted by centrifuging and stored at −20 °C. Results from three repeated cultures over 3 weeks were compared with a single culture and used for the 2-way contingency table to evaluate a variety of standard statistical measures for diagnostics tests accompanied by 95% confidence interval (95% CI) (Fleiss *et al*. 2013). Positive samples with trypanosomes were subcultured on a fresh biphasic medium. Non-contaminated isolates were stored in the cryobank of the Parasitology Laboratory, Faculty of Veterinary Science, The University of Sydney, Sydney, Australia.

Transmission electron microscope

Mid-exponential phase parasites were collected from culture and fixed in 2·5% glutaraldehyde (v/v) (PST, Queensland, Australia) in 0·1 M sodium cacodylate buffer (pH = 7·2) at 25 °C for 1 h. Fixed cells were washed in 0·1 M cacodylate buffer (pH = 7·2) and post-fixed in 1% (w/v) OsO_4 in 0·1 M sodium cacodylate buffer (pH = 7·2). The cell pellet was further processed through an ethanol dehydration series carried out at 25 °C and samples were infiltrated and embedded in Epon resin (PST, Queensland, Australia). Ultrathins were obtained using a diamond knife and mounted on grids. Subsequently, grids were stained with 2% uranyl acetate followed by 1% lead citrate. The grids were viewed and imaged on a JOEL JEM 1400 TEM, using 120 kV, equipped with an ES500W Erlangshen CCD camera (Gatan, California, USA) and the kinetoplast ultrastructure analysed as described by (Lukeš and Votýpka, 2000).

Amplification of the small subunit rRNA and glycosomal glyceraldehyde phosphate dehydrogenase genes

Total DNA was extracted from *Trypanosoma* culture pellets using Isolate II Genomic DNA kit (BioLine, Australia). DNA was eluted into 100 μL of Tris buffer (pH = 8·5) and stored at −20 °C. Small subunit rRNA gene (SSU rDNA, ~2100 bp) was PCR amplified using the *Trypanosoma*-specific primers: [S0359] S762 (5′-GAC TTT TGC TTC CTC TAW TG-3′)/[S0360] S763 (5′-CAT ATG CTT GTT TCA AGG AC-3′) (Sehgal *et al*. 2001). Reactions of 30 μl contained MyTaq Red Mix (BioLine, Australia), and approximately 5 ng of genomic DNA template (2 μl). For selected SSU rDNA genotypes we PCR amplified partial glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH, ~900 bp). The gGAPDH was amplified using seminested PCR with *Trypanosoma*-specific primers: primary PCR [S0562] GAPDHF (5′-CTY MTC GGN AMK GAG ATY GAY G-3′)/[S0563] GAPDHR (5′-GRT KSG ART ADC CCC ACT CG-3′), followed by a secondary PCR [S0562] GAPDHF/[S0565] G4a (5′-GTT YTG CAG SGT CGC CTT GG-3′) as previously described (Hamilton *et al*. 2004; McInnes *et al*. 2009). The cycling for SSU rDNA was as follows: denaturing at 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s, 53 °C for 15 s, 72 °C for 60 s, and a final elongation for 5 min at 72 °C. The gGAPDH primary cycling included denaturing at 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s and a final elongation for 5 min at 72 °C. The gGAPDH secondary cycling included denaturing at 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s, 52 °C for 15 s, 72 °C for 15 s and a final elongation for 5 min at 72 °C; 0·5 μL of the primary reaction was used as template for the secondary PCR. The PCRs were conducted in a PCR cycler Veriti (Life Sciences, Australia). All PCRs were run with a negative control of sterile PCR-grade water. Resulting products were resolved in 1% (w/v) agarose. All PCR that yielded product of an expected size (~2100 bp for SSU rDNA, ~900 bp for gGAPDH) were directly and bi-directionally sequenced using amplification primers and internal primers at Macrogen Ltd. (Seoul, Korea). Sequences were assembled, aligned with related sequences and analysed using CLC Main Workbench 6·9 (CLC bio, Denmark) and deposited in GenBank (National Center for Biotechnology Information, NCBI) under the Accession Numbers: KT728373-KT728402, KU323390-KU323394.

Phylogenetic analysis of the small subunit rRNA gene

Evolutionary analyses were conducted in MEGA 6·06 (Tamura *et al*. 2013) and MrBayes 3·2·5 (Ronquist *et al*. 2012). Multiple sequence alignment was constructed with available SSU rDNA sequences belonging to defined clades as described by Zídková *et al*. (2012). Multiple sequence alignment of SSU rDNA and gGAPDH is available as Supplementary Dataset 1 and 2. Sequence divergences were calculated using the Kimura 2 parameter (K2P) distance model, and phylogenetic tree was inferred using Minimum Evolution and the bootstrap support inferred from 1000 replicates in MEGA. Maximum likelihood fits for 24 different nucleotide substitution models was calculated in MEGA and K2P with a discrete gamma distribution evolutionary rate differences among sites with five categories (+G, parameter = 0·2497) and with the rate variation model allowing for some sites to be evolutionarily invariable (+I, 41·65%). The best fit model was based on the lowest BIC scores (Bayesian Information Criterion). The Maximum Likelihood was calculated with bootstrap support from 100 replicates in MEGA. For the Bayesian phylogenetic analysis using MrBayes we used K2P+G+I model. Metropolis-coupled Markov chain Monte Carlo analyses were run with one cold and three heated chains (temperature set to default 0·2) for 2 000 000 generations and sampled every 200 generations. This process was performed twice from a random starting tree and ran well beyond convergence. Trees before convergence (25% burnin) were discarded for the reconstruction of the consensus Bayesian tree with posterior probabilities.

RESULTS

Parasite description and identification

Parasitological examination of blood parasites from the Regent Honeyeaters revealed trypomastigotes of the

genus Trypanosoma Gruby, 1843, whereas haemosporidians were not detected and microfilariae were detected in only a single bird. The haemozoic trypomastigote stage is traditionally used for new species description and remains a significant component because they are conserved in different hosts (Votýpka and Svobodová, 2004; Valkiūnas *et al*. 2011). Morphological and morphometric characters of the haemozoic trypomastigotes are readily used in co-infections to differentiate *Trypanosoma* species (Molyneux and Gordon, 1975). Besides the specific guidelines for trypomastigote circumscription, it is emphasized that endogenous development, ultrastructure of the kinetoplast and ecological parameters should be included whenever possible with the species description (Wallace *et al*. 1983; Wheeler *et al*. 2013; Votýpka *et al*. 2015). Morphological, genetic and parasite culture investigations showed that this parasite represents a new species, the description of which follows.

Order Trypanosomatida Kent, 1880
Family Trypanosomatidae Doflein, 1901

Trypanosoma thomasbancrofti **sp. n.**

Diagnosis: Trypomastigotes scarce in thin blood films (Fig. 1). No more than 10 trypomastigotes recorded per blood film. Moderate size trypomastigotes, length 38·3 (27·6–43·4) μm, and moderately broad through the centre of their nuclei 4·6 (3·5–5·7) μm ($n = 20$) (Table 1). Moderately even breadth of the middle third of the trypomastigote (Fig. 1). Oval nucleus positioned centrally, the anterior part, 15·0 (13·3–17·7) μm, and posterior 13·6 (10·4–16·6) μm parts measured from the centre of the nucleus approximately even size (Table 1). The kinetoplast small and oval, not clearly stained using Diff-Quick stain. The kinetoplast 5·0 (2·9–7·1) μm from the posterior end ($n = 20$). The posterior end around the kinetoplast stained poorly compared to the deeply stained remainder of the trypomastigote ($n = 20$). The anterior end more markedly drawn out and pointed than the posterior end. Longitudinal striations observed in some trypomastigotes, particularly over the centrally positioned nucleus. In areas of deeply stained cytoplasm located outside the nucleus, striations were indistinct. The undulating membrane, pale with distinct outline, formed by the stained flagellum throughout the trypomastigote. Free flagellum originates just anterior to the kinetoplast. No dividing forms seen. No information regarding pathogenicity in birds, trypanosomes detected in clinically healthy birds. Ultrastructural investigation of the epimastigotes of *T. thomasbancrofti* sp. n. originated from a culture showed a kinetoplast thickness 549·4 (490·5–685·1) μm (Fig. 2).

Discovery of T. thomasbancrofti *sp. n. in the Regent Honeyeater*

Initial blood smear observation of two birds (B20248, B20358) demonstrated scarce presence of trypomastigotes belonging to the genus *Trypanosoma* (B20248, <30 and B20358, <5 per blood smear, March 2013) (Fig. 1). Subsequently, inoculation of the blood onto a biphasic media revealed presence of epimastigotes in culture B20358 (RHE/B20358/AUS/2013/TB1) from 5 days onward (Regent Honeyeaters, Cohort 1; Supplementary Table S1). Amplification and direct PCR amplicon sequencing of SSU rDNA with *Trypanosoma*-specific primers demonstrated presence of a 2105 nt sequence with top blastn hits belonging to passerine bird *Trypanosoma* spp. sequences. Culture B20248 did not reveal the presence of epimastigotes (28 days). Ultrastructural investigation of the epimastigotes of *T. thomasbancrofti* sp. n. (B20358) based on TEM showed a distinct kinetoplast thickness that measured 549·4 μm (490·5–685·1, S.D. 44·7, $n = 24$) (Fig. 2).

We then tested six birds for the presence of *T. thomasbancrofti* sp. n. using repeated blood sampling tested by culture (April/May 2013; Regent Honeyeaters, Cohort 2; Supplementary Table S1). Blood was inoculated onto a biphasic medium three times, following a 7-day interval. Epimastigotes were detected as early as 4 days after inoculation. Blood from B20358 was culture positive on all three attempts. Blood from B20331 was culture positive on a single occasion, 6 days post inoculation, with low numbers of clumping epimastigotes not observed on subsequent examinations. The remaining blood cultures were negative. We then used the repeated blood *Trypanosome* culture approach on six birds as the gold standard test for a 2-way contingency table. Sensitivity (Se) and specificity (Sp) of single *Trypanosome* culture was 50% (95% CI, 3–50%) and 100% (76–100%), respectively. Positive predictive value (PPV) was 100% (5·7–100%) and negative predictive value (NPV) was 80% (61–80%) for a single blood culture.

Overall, the initial *Trypanosoma*-prevalence was 29·4% (5/17) in captive birds based on culture tests during 2013/2014 (B20213, B20217, B20331, B20358, B30214; Supplementary Table S1). In 2013, seven birds were tested using single culture attempt, where two birds revealed the presence of *Trypanosoma* sp. (July, 2013; Regent Honeyeaters, Cohort 3; Supplementary Table S1). In 2014, six birds were tested using single culture attempt and one bird (B30214) revealed presence of trypanosomes, 8 days post inoculation (October/November, 2014; Regent Honeyeaters, Cohort 4; Supplementary Table S1), subsequent sequencing confirmed *T. thomasbancrofti* sp. n. Comparison of the SSU rDNA of these two *T. thomasbancrofti* sp. n. genotypes revealed 6 nucleotide/in-del differences between the genotype g1 (culture RHE/B20358/AUS/2013/TB1; GenBank: KT728373) and the genotype g2 (culture RHE/B30214/AUS/2014/TB2; GenBank: KT728395).

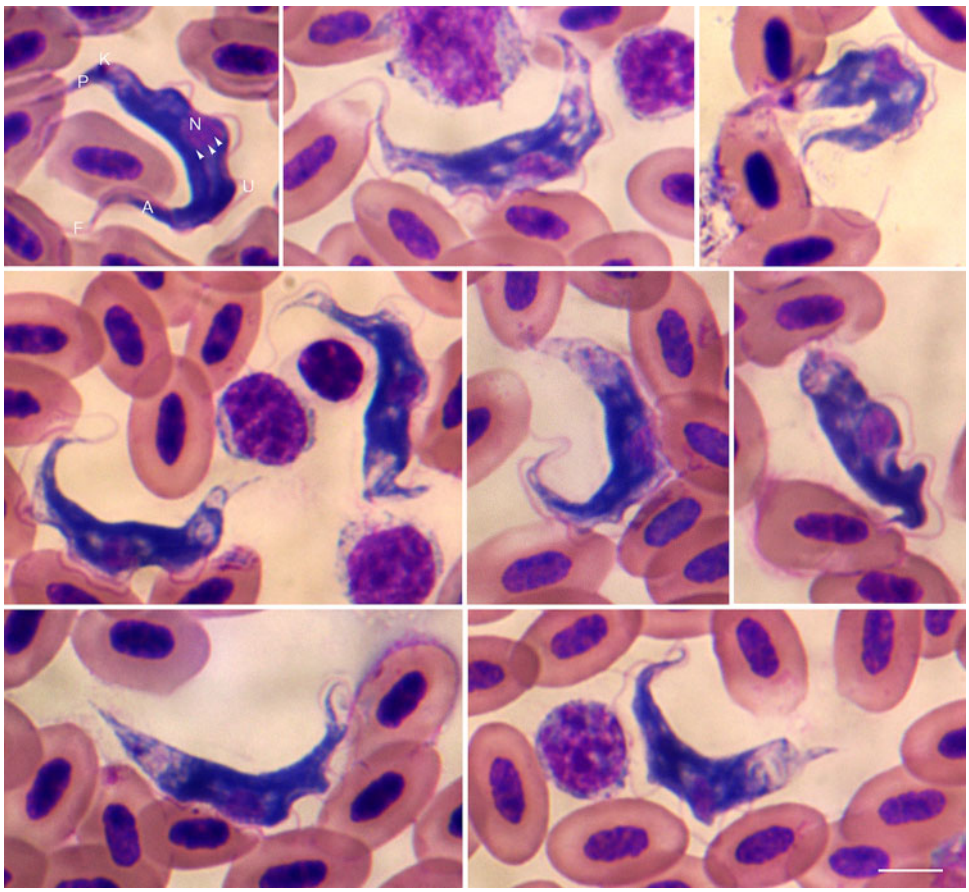

Fig. 1. *Trypanosoma thomasbancrofti* sp. n. (Kinetoplastida) on a blood smear from the Regent Honeyeater. Moderately broad trypomastigotes possess an oval nucleus (N) positioned centrally. The kinetoplast (K) is small and oval. Free flagellum (F) originates just anterior to the kinetoplast. The anterior end (A) more markedly drawn out and pointed than the posterior end (P). Longitudinal striations (arrows) observed in some trypomastigotes, particularly over the centrally positioned nucleus, in areas of deeply stained cytoplasm outside the nucleus striation are indistinct. The undulating membrane (U) is visible throughout the trypomastigote. Composite image, all at the same magnification. Scale bar, 5 μm. Diff-Quick stain. Type material G466177 at the Queensland Museum, Australia (from a type host ID: B20248 at Taronga Zoo).

In 2015, Regent Honeyeaters considered for release ($n = 81$) were screened for the presence of *Trypanosoma* spp. using culture followed by SSU rDNA PCR and direct sequencing. From 81 birds screened, 27 birds (33·3%, 27/81) were found to be culture positive for trypanosomes (Supplementary Table S2). Only a single blood smear revealed scarce presence of (<2) trypomastigotes (B40305). Dividing the birds into age groups based on date of birth, we demonstrated increased prevalence with age (Fig. 3). Birds born in 2014 (age 1 year) had prevalence of 24·4% (10/41), birds born in 2013 (age 2 years) had prevalence of 37·5% (12/32) and birds born 2012 (age 3 years) had prevalence of 57·1% (4/7). A single 5-year-old bird born in 2010 (B30152) was *Trypanosoma* positive. DNA was isolated from all 27 *Trypanosoma*-positive cultures and SSU rDNA was amplified from 25 samples. SSU rDNA sequences matching *T. thomasbancrofti* sp. n. were amplified in 20 samples (20/25), and found to be identical to SSU rDNA sequence from B20358 (genotype g1) collected in 2013 (RHE/B20358/AUS/2013/TB1; GenBank: KT728373). In fact, one of the 20 samples was from the same B20358 bird, confirming that *T. thomasbancrofti* sp. n. remains genetically stable (genotype g1) over a 2-year period. For the sample from B20358, we isolated DNA from the culture, 3 and 6 days post inoculation, and confirmed that only unambiguous SSU rDNA of *T. thomasbancrofti* sp. n. was present. Five SSU rDNA sequences (from B20348, B30187, B30238, B40102, B40384) were distinct from *T. thomasbancrofti* sp. n., nevertheless the top blastn hits belonged to passerine bird *Trypanosoma* cf. *avium* SSU rDNA sequences. Four SSU rDNA sequences were identical to each other (B20348, B30187, B30238, B40102) and constitute genotype g3 with 18 nucleotide/in-del differences from *T. thomasbancrofti* sp. n. SSU rDNA sequences. Sequence from B40384 represents *Trypanosoma* cf. *avium* genotype g4 and differ in 12 nucleotide/in-del from genotype g3 (Supplementary Table S2).

In total, we have tested 92 individual captive birds (Supplementary Tables S1 and S2). Five birds were

Table 1. Morphology of trypomastigotes of *Trypanosoma thomasbancrofti* sp. n

Feature	Mean (minimum, maximum)	SD
area of kinetoplast (AK)	0·7 (0·5, 1·1) μm	0·2 μm
area of nucleus (AN)	3·9 (3·2, 4·7) μm	0·4 μm
area of trypomastigote (AT)	38·3 (27·6, 43·4) μm	3·5 μm
width of body through centre of nucleus (BW)	4·6 (3·5, 5·7) μm	0·6 μm
free flagellum (FF)	8·3 (6·4, 11·7) μm	1·7 μm
kinetoplast to centre of nucleus (KN)	10·0 (7·4, 13·5) μm	1·7 μm
centre of nucleus to anterior end (NA)	15·0 (13·3, 17·7) μm	1·3 μm
total length without free flagellum (PA)	27·2 (19·5, 31·5) μm	3·4 μm
posterior end to kinetoplast (PK)	5·0 (2·9, 7·1) μm	1·0 μm
posterior end to centre of nucleus (PN)	13·6 (10·4, 16·6) μm	1·7 μm
AN/AT	0·10	0·11
BW/PA	0·17	0·17
PK/PA	0·18	0·29
PN/KN	1·35	1·01
PN/NA	0·90	1·28
PN/PA	0·50	0·49

S.D., standard deviation.

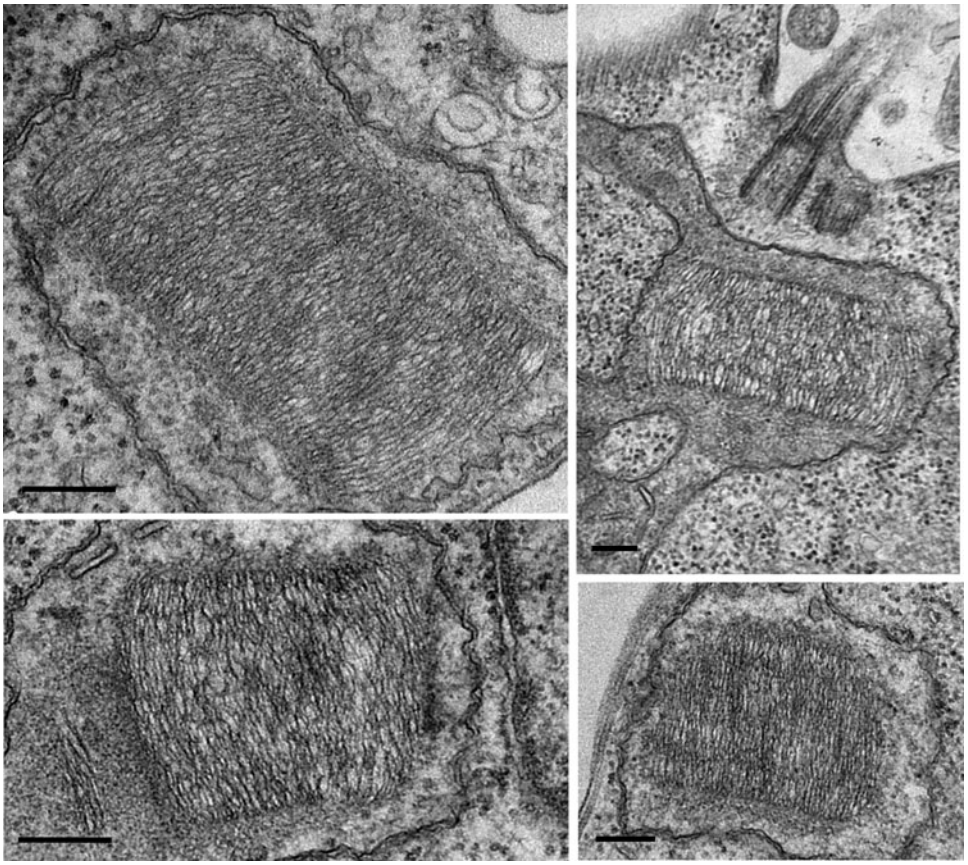

Fig. 2. Transmission electron microscopy of kinetoplasts from cultured *Trypanosoma thomasbancrofti* sp. n. (Kinetoplastida). Characteristic cylindrical-shaped kinetoplasts are represented by isolate RHE/B20358/AUS/2013/TB1 (genotype g1). All micrographs are to the same scale. Scale bar, 5 μm.

tested in 2013/2014 and subsequently in 2015, testing *T. thomasbancrofti* sp. n. positive with SSU rDNA sequence matching RHE/B20358/AUS/2013/TB1. Only birds which maintained or gained trypanosomes were recorded, no birds that cleared trypanosomes were recorded. Bird B20358 remained positive from 2013 to 2015. Birds B20332 and B20390 were negative on three occasions in 2013, but positive in the 2015 screening. Moreover, B20390 tested negative in 2014. Birds B30403 and B30404 were negative in 2014, but positive in 2015.

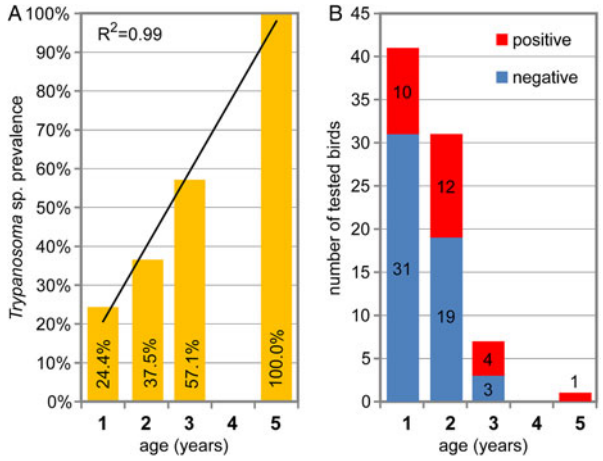

Fig. 3. Bird age correlation with prevalence of trypanosomes in the Regent Honeyeater at the Taronga Zoo. Bird blood samples were collected and cultured to detect presence of trypanosomes. (A) Birds ($n = 81$) were divided into age groups and their prevalence for evaluation of correlation (coefficient of determination, R^2). (B) The graph shows total number of birds tested born in an individual year, each bar is divided according to the trypanosome diagnostic results.

Following the release in 2015, we collected blood samples from 18 Regent Honeyeaters captured at the release site and screened for the presence of *Trypanosoma* spp. using culture, followed by SSU rDNA PCR and direct sequencing. From the 18 birds screened, four birds (22·2%, 4/18) were culture positive for *Trypanosoma* (Supplementary Table S3). Two blood smears revealed scarce presence of (<2) trypomastigotes (B20305, B20239) and only one (B20239) was later positive in culture. Amplification and sequencing of SSU rDNA demonstrated presence of the *T. thomasbancrofti* sp. n. genotype g1 in two birds (B40304, B40442) and *T. thomasbancrofti* sp. n. genotype g2 in a single bird (B40584), the *T.* cf. *avium* genotype g3 in a single bird (B20239). Three of these birds, (B40584, B40304 and B40442), tested negative for *Trypanosoma* spp. in 2015 prior to release, suggesting that these birds acquired the infection in the wild. Seven tested negative in the wild, but tested positive while captive at Taronga Zoo. A single wild bird tested negative for trypanosomes.

Trypanosoma *cf.* avium *in the Noisy Miner (*M. melanocephala*)*

To investigate the possibility that *T. thomasbancrofti* sp. n. from Regent Honeyeaters is also present in other bird species, we collected blood from Noisy Miners, captured in close proximity to Regent Honeyeater aviaries at Taronga Zoo. Blood smears from nine birds revealed no hemozoic trypomastigote, a single bird had intraerythrocytic *Haemoproteus* sp. (#6) and three birds, (#2, #5, #7), had microfilaria present. Blood collected from 11 birds (May 2013) was inoculated onto the biphasic medium as pooled samples from two birds per culture flask. Trypanosomes were demonstrated in three pools on day 6 post inoculation (Supplementary Table S1). Subculturing of the trypanosomes onto a fresh media was unsuccessful and the trypanosomes were not observed in the cultures by day 28 post inoculation.

DNA was isolated from the culture from Noisy Miner pool #9/#10 (NM/#9#10/AUS/2013/TA1), followed by PCR amplification with *Trypanosoma*-specific primers confirmed presence of *Trypanosoma* sequence (SSU r|DNA, 2099 nt). Querying the new sequence against publically available trypanosome sequences confirmed high similarity with *Trypanosoma* from passerine birds and 100% identity with the *Trypanosoma* cf. *avium* SSU rDNA sequences (genotype g3) from the Regent Honeyeaters (B20348, B30187, B30238, B40102).

Phylogeny of bird Trypanosoma *species*

For the purpose of the phylogeny we define four distinct and newly defined *Trypanosoma* SSU rDNA genotypes (g1–g4) (Fig. 4). Phylogenetic inference of SSU rDNA demonstrates that g1–g4 clustered within Group C *sensu* Zídková *et al.* (2012) and the overall phylogeny was comparable with previously published trees (Zídková *et al.* 2012) (Fig. 4). Both closely related SSU rDNA sequences (g1, g2) of *T. thomasbancrofti* sp. n. obtained from 24 Regent Honeyeaters clustered with closely related sequences belonging to an unnamed *Trypanosoma* sp. in the Group C-II *sensu* Zídková *et al.* (2012) (Fig. 4). The sequences of *Trypanosoma* cf. *avium* (g3) from one Noisy Miner and four Regent Honeyeaters were a sister group to the *T. avium* sequences in the Group C-X + XI *sensu* Zídková *et al.* (2012) (Fig. 4). The single SSU rDNA sequence (g4) from one Regent Honeyeater (B40384; RHE/B40384/AUS/2015/TA2) was almost identical (one mismatch over 2102 nt, 99·95% identity) to a published *Trypanosoma* sp. isolate N335 from the Java Sparrow (*Padda oryzivora*) (AJ223570) (Haag *et al.* 1998). For birds in Australia, Hamilton *et al.* (2004) published a *Trypanosoma* sp. SSU rDNA sequence (AJ620557) from the Australian Currawong (*Strepera* sp.), however, it is clearly distinct from all our new *Trypanosoma* spp. sequences and belongs to the Group B *sensu* Zídková *et al.* (2012) (Fig. 4). Currently, no other sequences from Australian bird trypanosomes are publically available for comparison.

The alignment of the Group C-II, including isolates PAS105, PAS112, PAS106 from the Common Chiffchaff (*P. collybita*), CUL15 from the common house mosquito (*C. pipiens* complex)

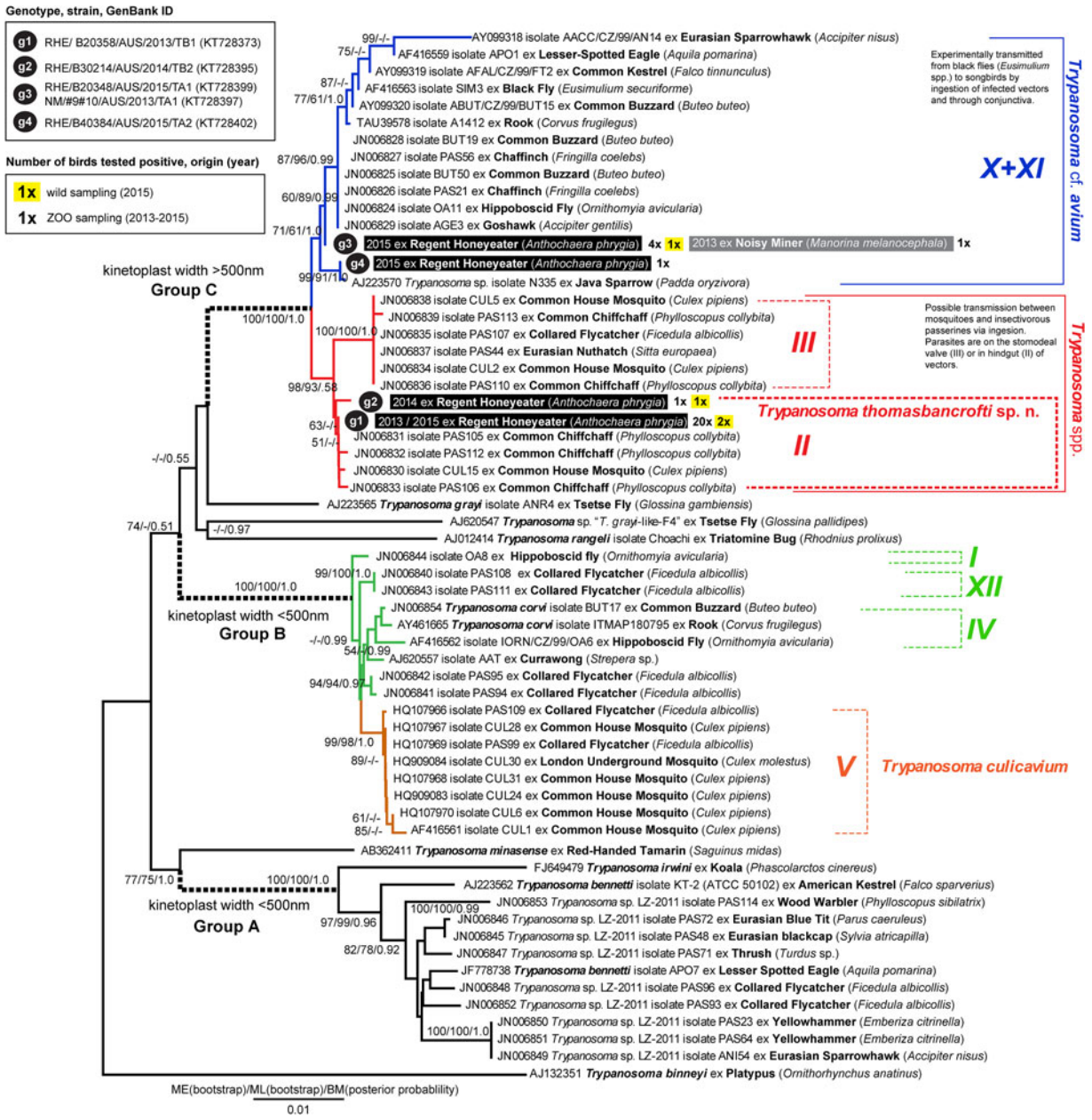

Fig. 4. Phylogenetic tree of SSU rDNA sequences of avian *Trypanosoma* species. The tree was inferred using the Minimum Evolution [ME, Kimura 2-parameter (K2P) distances] method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test using ME (1000 replicates) and Maximum Likelihood (ML, 100 replicates), and Bayesian Method (BM, posterior probability) are shown next to the branches. The ML model (K2P) included a discrete gamma distribution and allowed for invariable sites. The BM was based on K2P model with estimated discrete gamma distribution and proportion of invariable sites. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Branch annotation (groups A–C) and clusters within 'clade C' are according to Zídková *et al*. (2012). *Trypanosoma binneyi* served as an outgroup.

and both *T. thomasbancrofti* sp. n. genotypes (2108 residues) from Regent Honeyeaters revealed only nine singletons with overall distance of 0·001. Such high overall sequence identity (99·57%) of the group suggests that all belong to the same species, despite the fact that the Common Chiffchaff and the common house mosquito were collected from Central Europe (Czech Republic). A possible transmission between mosquitoes and insectivorous passerines by ingestion was proposed for the Group C-II/III with parasites located on mosquito stomodeal valves (Group C-III) or in the hindgut (Group C-II) of vectors (Votýpka and Svobodová, 2004; Zídková *et al*. 2012).

The phylogenetic clades of avian *Trypanosoma* species based on gGAPDH gene trees share common origin except *T. bennetti* (Hamilton *et al*. 2004; McInnes *et al*. 2009). Five *Trypanosmoma* isolates were further characterized at gGAPDH locus (Supplementary Fig. 1). Three isolates with SSU

rDNA sequences (g1) of *T. thomasbancrofti* sp. n. from Regent Honeyeaters were identical to each other or differed by a single nucleotide difference (B20358, B30188, 11006). The isolate with SSU rDNA sequences (g2) of *T. thomasbancrofti* sp. n. (11008) was 96% identical at gGAPDH from those isolates represented by SSU rDNA sequences (g1). An isolate belonging to *T.* cf. *avium* from a Regent Honeyeater (B30187, SSU rDNA sequence – g3) was 92% identical to *T. thomasbancrofti* sp. n. at gGAPDH.

DISCUSSION

The present study describes a new trypanosome species, *T. thomasbancrofti* sp. n., isolated from the endangered Regent Honeyeater. The newly described trypanosome falls within the Group C-II *sensu* Zídková *et al.* (2012) of avian trypanosomes sharing high (>99%) SSU rDNA sequence identity. One of the most conspicuous features of trypanosomes is its compact mitochondrial DNA forming a characteristic kinetoplast (Yurchenko *et al.* 1999; Votýpka *et al.* 2002). The thickness of the kinetoplast in avian trypanosomes was suggested as a characteristic feature of distinct species and lineages (Votýpka *et al.* 2012; Zídková *et al.* 2012). The kinetoplast thickness 549±45 (491–685) μm of *T. thomasbancrofti* sp. n. is near identical to Group C-II isolates CUL15 and PAS112 (546; 458–668 μm) and PAS112 (527; 446–676 μm), respectively (Zídková *et al.* 2012). The high sequence identity (>99·5%) and the kinetoplast ultrastructure of *T. thomasbancrofti* sp. n. are the key differentiating characteristics from other *Trypanosoma* spp. Twenty four newly obtained sequences forming two genotypes (g1 and g2) clustered along with the four previously described isolates (PAS105, PAS106, PAS112 and CUL15) of the Group C-II *sensu* Zídková *et al.* (2012) formed a monophyletic group representing the new species, *T. thomasbancrofti* sp. n. None of the named species of *Trypanosoma* spp. could be directly associated with *T. thomasbancrofti* sp. n.

Our findings support the opinion of Valkiūnas *et al.* (2011), stating the authors' early descriptions that avian trypanosomes are specialists and therefore highly bird host specific is incorrect and such labelled species need careful re-evaluation. In the case of the endangered Regent Honeyeater, the ultrastructure and DNA sequence strongly suggests that *T. thomasbancrofti* sp. n. is not geographically restricted and host specific, but likely, an intercontinentally distributed generalist trypanosome species. As such, we propose to adopt the name for the entire Group C-II that includes trypanosomes isolated from the Common Chiffchaff (*P. collybita*) distributed throughout Europe, Asia and Africa. Generally, there is little evidence of host–trypanosome specificity, because identical or close to identical SSU rDNA sequences of bird trypanosomes are amplified from diverse birds over large geographical distances (Sehgal *et al.* 2001; Votýpka and Svobodová, 2004; Zídková *et al.* 2012). The mechanisms of trypanosome ecological plasticity warrants the attention of parasitologists and evolutionary biologists, because our example of closely related trypanosome SSU rDNA sequences from Australia's Regent Honeyeater and Europe's Common Chiffchaff suggests an intercontinental distribution.

One of the more challenging aspects encountered when working with trypanosomes is their diagnosis in avian hosts. Stained blood smears are notoriously ineffective; therefore more sensitive culture and PCR are used in studies examining trypanosome diversity (Sehgal *et al.* 2001; Votýpka *et al.* 2012; Zídková *et al.* 2012). To evaluate reliability of a single blood sampling to detect trypanosomes via culture, a technique adopted throughout this study, we tested birds weekly over a 3-week period. Comparison of a diagnostic culture against PCR would be an alternative approach, but again the analysis would be incomplete due to the absence of true positive and negative birds. The low sensitivity with high specificity of using culture as diagnostics presented here requires careful interpretation because only six birds were used in the repeated culturing experiment due to ethical and logistical considerations. Culture technique can theoretically return positive result even if a single trypanosome is present in the inoculum (50 μL of fresh avian blood). The higher NPV of 80% demonstrates the sensitivity of returning a negative result in the absence of trypanosomes in the inoculum. On the other hand, the wide 95% CI of the positive predictive value (PPV) may reflect either extremely low parasitaemia of a persistent infection or only a transient infection that did not establish. At this time we have no means to distinguish between the two.

This study revealed presupposed association of bird age with increased prevalence of *Trypanosoma* spp. The longest living captive Regent Honeyeaters have reportedly lived for 17 years, nevertheless the realistic life expectancy is 12–14 years (unpublished data, Larry Vogelnest). *Trypanosoma* species are common parasites of birds, yet the parasitaemia is often extremely low to be detected on blood smear compared with blood culture examination shown to be 20 times more sensitive in the case of the Regent Honeyeaters. The knowledge on the persistence of *Trypanosoma* spp. in birds is incomplete. On one hand, we demonstrated that a single bird was infected with the same genotype of *T. thomasbancrofti* sp. n. over a 2-year period, thereby arguing for persistence of infection. On the other hand, in our data from recaptured birds following release,

we noted a number of birds that possibly eliminated parasitaemia based on results from culture testing. Birds that potentially eliminated pre-release parasitaemia possessed both *T. thomasbancrofti* sp. n. (genotypes 1, 2) as well as *T.* cf. *avium* (genotype 3) suggesting that the bird immune system could clear the infection. Alternatively, the infection was below the detectable level of the culturing technique. Repeated testing was not possible to confirm either elimination or persistence. Sampling of the released birds demonstrated that birds which tested negative prior to release acquired *Trypanosoma* spp. in the wild. The data confirm that *T. thomasbancrofti* sp. n. is present at the site of release. The phenomenon of elimination *vs* persistent *Trypanosoma* infection will require further experimental scrutiny. It remains to be elucidated whether parasitaemia is a good indicator of the presence of *Trypanosoma* spp. in the bone marrow.

In Australia, birds are known hosts of *Trypanosoma* spp., yet their diversity is unknown as studies applying molecular tools are absent (O'Donoghue and Adlard, 2000). Two named species have been described to date, *T. anellobiae* and *T. myzanthase*, based on trypomastigotes from bird blood smears (Cleland and Johnston, 1910; Mackerras and Mackerras, 1960; Bennett *et al.* 1994*c*). The original description of *T. anellobiae* is from the Little Wattlebird (*Anthochaera chrysoptera*), but it was later re-described from the Noisy Miner (Bennett *et al.* 1994*c*). Besides these named species, several trypomastigote forms are described, but their further taxonomic status is not defined (Cleland and Johnston, 1911; Breinl, 1913; Mackerras and Mackerras, 1960). Mackerras and Mackerras (1960) reviewed all previously illustrated forms of trypomastigotes recorded from Australian birds, but could not ascertain how to classify *Trypanosoma* spp. from different bird species or families, because of unknown host specificity. In general, *Trypanosoma* species identification, merely through trypomastigote morphology, is in most cases regarded as insufficient. *Trypanosoma* species in birds in Australia are no different to those from other continents where the majority of original descriptions based on trypomastigote morphology are difficult or even impossible to use for species identification and comparisons. Nevertheless, *T. anellobiae* and *T. myzanthas*, differ primarily in the position of their kinetoplast and were distinguished as follows '*T. myzanthae* differs from *T. anellobia* in shape, being usually broader, in the position of undulating membrane, usually wound around the body, in the central position of the nucleus, and in the longer, thicker, free flagellum' (Mackerras and Mackerras, 1960). Trypomastigotes of *T. anellobia* are narrow and approximately 35 μm long, displaying kinetoplasts 3 μm from the posterior end (Cleland and Johnston, 1910). The broader trypomastigotes of *T. myzanthae* (19–25 μm) have kinetoplasts <1·5 μm from the posterior end (Mackerras and Mackerras, 1960). Based on the original morphological description of *Trypanosoma* spp. alone, these were later considered ambiguous. The deteriorated status of the type slide held at the Australian Museum, deposited by Cleland and Johnston (1910), led Bennett *et al.* (1994*c*) to designate a neotype. The neotype of *T. anellobia* was described from the Noisy Miner and is considered conspecific with the original type material from the Little Wattlebird despite the trypomastigote size averaging 25·6 (S.D. 2·3) μm (Bennett *et al.* 1994*c*). The above seemingly complicates the situation of trypanosomes in Australian birds, because the Noisy Miner is the type host of the *T. myzanthae* described by Mackerras and Mackerras (1960). It is likely that Noisy Miners are hosts to more than one species of trypanosome and isolation of these trypanosomes is considered necessary to resolve the relationship to trypanosomes from Regent Honeyeaters and advance the intercontinentally growing database of bird *Trypanosoma* SSU rDNA sequences.

We cultured and sequenced SSU rDNA of *T.* cf. *avium* from the Noisy Miners and Regent Honeyeaters. Based on the above, there are at least three reasons why the situation in Noisy Miners as the type hosts for both *T. anellobia* and *T. myzanthae* presents a confusing case. (i) *Trypanosoma myzanthae* is not considered in a differential diagnosis of the neotype *T. anellobia* by Bennett *et al.* (1994*c*). (ii) We were not able to detect any trypomastigotes in our set of blood smears from Noisy Miners to perform morphological comparison to *T. anellobia* and *T. myzanthae*. (iii) The SSU rDNA sequence obtained from Noisy Miners suggests a close relationship with *T. avium* group. Historically, the *T. avium* group contained trypanosomes of owls and raptors, nevertheless, songbirds were later confirmed as the hosts of *T. avium* (Baker, 1976; Podlipaev, 1990; Dirie *et al.* 1991; Votýpka *et al.* 2002; Zídková *et al.* 2012). A member of the *T. avium* group was experimentally transmitted from black flies (*Simulium*/*Eusimulium* spp.) to songbirds by ingestion of infected vectors and through conjunctiva (Votýpka and Svobodová, 2004). In Australia, old records of suspect *T. avium* group trypanosomes exist for the Brahminy Kite (*Haliastur indus girrenera*), the Grey Falcon (*Falco hypoleucos*) and the Boobook Owl (*Ninox boobook*) (Breinl, 1913; O'Donoghue and Adlard, 2000). Currently, none of these owl or raptor trypanosomes are available for comparison with either our new sequences or the intercontinentally growing database of SSU rDNA (Votýpka *et al.* 2002; Votýpka and Svobodová, 2004; Zídková *et al.* 2012). It is likely, that *T. avium* complex species exist in Australia, because it infects a broad range

of avian hosts and has been suggested to be intercontinentally distributed (Bennett, 1961; Votýpka *et al*. 2002; Zídková *et al*. 2012). It was noteworthy that we did not detect dual infection in any of the studied cultures. Whether absence of dual infection was an artefact of the culture is not known. It remains to be elucidated if birds infected with one trypanosome remain resistant to a super-infection with a different trypanosome.

The current results demonstrate that the screening approach applied to the captive bird population is suitable for screening of wild birds. Single culture was sufficient to demonstrate parasitaemia during the repeated culture experiment and is therefore recommended for wild bird screening. Additional genotyping of cultured trypanosomes from Regent Honeyeaters will be required to demonstrate conspecificity with *T. thomasbancrofti* sp. n. and absence of other *Trypanosoma* spp. belonging to other bird clades in either wild or captive birds.

Currently, no information exists on the effect of *T. thomasbancrofti* sp. n. on its hosts; however, all five trypanosome positive captive birds remained clinically healthy for over 12 months (Vogelnest, personal communication). Nevertheless, an argument that infected birds should not be released because the trypanosome species present in captive birds might be alien has been considered. If it is alien, the parasite might give rise to disease in free-living birds. However, given the limited evidence of pathogenicity and the worldwide distribution of Clade C-II and therefore *T. thomasbancrofti* sp. n. there is insufficient evidence to justify not releasing positive birds. The finding that at least some birds acquired *T. thomasbancrofti* sp. n. infection after their release demonstrates natural trypanosome circulation in their wild environment. The revelation that both the free living Noisy Miners and captive as well as wild Regent Honeyeaters serve as hosts of an identical *T.* cf. *avium* confirms previous suggestions that *T. avium* complex are capable of infecting wide range of birds including passerines (Bennett and Fallis, 1960; Bennett, 1961; Bennett *et al*. 1994*a*, *b*; Votýpka *et al*. 2002; Votýpka and Svobodová, 2004; Votýpka *et al*. 2012; Zídková *et al*. 2012).

We suggest that *T. thomasbancrofti* sp. n. has an intercontinental distribution. Current data are unable to identify whether the trypanosome is native or introduced into Australia. However, if the parasite is a generalist infecting migratory songbirds, then most likely, the parasite should be considered native to Australia. Alternatively, the parasite may have been introduced with invasive birds, such as the Common Myna (*Acridotheres tristis*), a known reservoir for avian malaria in Australia (Clark *et al*. 2015). In Australia, the Common Myna has not been extensively surveyed for trypanosomes, but a record of an unknown *Trypanosoma* sp. exists from its native Sri Lanka (Crusz, 1984). A small survey of Common Mynas in South East Queensland, Australia, showed no presence of trypanosomes in blood smears (N. Clark, personal communication). To evaluate if *T. thomasbancrofti* sp. n. was present in Australia prior the introduction of the Common Myna in the 19th century, we would need to consider testing preserved material from before such introduction. Museum materials of frogs have been successfully sampled to show that some myxozoan parasites in Australia were not present in frogs prior to the introduction of the Cane Toad (*Rhinella marina*) to Australia in 1935 (Hartigan *et al*. 2010). Trypomastigotes, however, are rare in birds' blood and do not concentrate in specific body cavities, thereby preventing its detection, unlike the myxozoa that accumulate in gallbladders of frogs. It is unlikely we can obtain conclusive evidence for the introduction of *T. thomasbancrofti* sp. n., retrospectively. Furthermore, museum material is unlikely to serve as a good proxy to evaluate absence of the parasite prior to introduction of exotic birds.

With almost one hundred individual birds tested for trypanosomes, we have tested the equivalent of one quarter of the remaining population of less than 400 wild Regent Honeyeaters. In conclusion, the trypanosomes in the Regent Honeyeaters represent the most intensive assessment of avian trypanosomes in Australia. However, the lack of baseline data on parasite diversity in Australia, such as diversity of *Trypanosoma* spp., limits our ability to ascertain the risk of whether parasites differ between source and destination sites and to what extent they may cause disease. It remains only an assumption that other Australian passerine birds are hosts for *T. thomasbancrofti* sp. n. either in the captive population or the remaining localities of the currently fragmented distribution of wild Regent Honeyeaters. The distribution of mosquitoes, in particular of the genus *Culex* (for example, *C. pipiens* group, *C. annulirostris*), as suspect vectors of *T. thomasbancrofti* sp. n. overlaps both captive and wild locations (Hemmerter *et al*. 2009; Russell, 2012). Ingestion of infected mosquitoes as the likely route of transmission makes any preventative measures complex, if not impractical, when considering captive breeding programmes.

Taxonomic Summary

Trypanosoma thomasbancrofti sp. n. Šlapeta
Trypanosomatida: Trypanosomatidae

Type host: Regent Honeyeater, *Anthochaera phrygia* (Shaw, 1794) (Aves: Passeriformes: Meliphagidae); syn. *Xanthomyza phrygia* (Shaw, 1794).

Type locality: Zoo breeding population at Taronga Zoo, Mosman, Sydney, New South Wales, Australia. Animals are living at Taronga Zoo or were released.

Site of infection: Blood plasma.

Type material/hapantotype: Blood smear from Regent Honeyeater, *Anthochaera phrygia* (ID: B20248, collected on 1 February 2013) deposited under accession number G466177 at the Queensland Museum, South Brisbane QLD 4101, Australia. Parahapantotypes: blood smears G466180 (ID: B40305, collected on 17 February, 2015) and G466181 (ID: B20358, collected on March 14, 2013).

The ZooBank LSID: urn:lsid:zoobank.org:act:D8949216-F714-4CCB-850D-CAE6884CC8EC

Prevalence: Using birds considered for release in 2015, 33·3% (27/81) bird bloods tested culture positive for *Trypanosoma* spp., and 24·4% (20/81) were successfully genotyped as *T. thomasbancrofti* sp. n. in the captive population at Taronga Zoo in 2015. Only a single blood smear, 1·2% (1/81), demonstrated presence of trypomastigotes.

Nucleotide sequence data: The sequence of the small subunit ribosomal RNA gene (SSU rDNA) and glycosomal glyceraldehyde phosphate dehydrogenase gene (gGAPDH) from the Regent Honeyeater (ID: B20358, currently at Taronga Zoo) is available in GenBank™ under Accession No. KT728373, KU323392 and designated as RHE/B20358/AUS/2013/TB1). Identical SSU rDNA of *T. thomasbancrofti* sp. n. was identified in an additional 19 and 2 Regent Honeyeaters, *A. phrygia,* held at Taronga Zoo and released into the wild, respectively (Supplementary Tables S2 and S3; GenBank: KT728374-KT728392, KT728393-KT728394). Near identical SSU rDNA (>99·5%) with *T. thomasbancrofti* sp. n. was identified in a single Regent Honeyeater, *A. phrygia* originated from Taronga Zoo and in one released wild bird (GenBank: KT728395).

Additional hosts: *T. thomasbancrofti* sp. n. was identified, based on the high sequence identity (>99·5%) with SSU rDNA of *T. thomasbancrofti* sp. n., in the Common Chiffchaff, *Phylloscopus collybita* (isolate PAS105, PAS112, PAS106, GenBank: JN006831, JN006832, JN006833) and from the Common House Mosquito, *Culex pipiens* complex (CUL15, GenBank: JN006830) in Central Europe.

Etymology: The specific epithet "*thomasbancrofti*" is given in honour from the surname of Thomas Lane Bancroft (1860–1933) who undertook many parasitological investigations in Australia and whom was the first to note trypanosomes in Australian endemic bird blood smears.

Remarks: This is the first *Trypanosoma* species described from the Regent Honeyeater (*A. phrygia*). In Australia, *Trypanosoma myzanthase* Mackerras and Mackerras, 1960 and *Trypanosoma anellobiae* Cleland and Johnston, 1910 are described from passerine (Passeriformes) birds and honeyeaters (Melliphagidae). The original description of *T. anellobiae* is from the Little Wattlebird (*A. chrysoptera*), a honeyeater from the same genus *Anthochaera*. Trypomastigote of *T. thomasbancrofti* sp. n., however, is distinct from both described species in shape and position of kinetoplast. *T. anellobiae* is very narrow and approximately 35 μm in length with kinetoplast 3 μm from the posterior end. *T. myzanthase* is broad, 19–24 μm in length, with rather large and distinct kinetoplast situated <1·5 μm from the posterior end. Our species shape is amid the two described species and the kinetoplast is further from the posterior end compared to *T. anellobiae* and *T. myzanthase*. Intercontinentally, there are several avian *Trypanosoma* spp. described from various bird groups including passerine (Passeriformes) birds. Absence of type material, insufficient illustration of type material and cryptic morphology make it difficult to compare and identify avian *Trypanosoma* species at an intercontinental scale. Molecular characterization enabled us to show that Regent Honeyeaters, *A. phrygia*, held at Taronga Zoo are hosts for four *Trypanosoma* spp. SSU rDNA genotypes. Two SSU rDNA genotypes (g1, g2) are considered *T. thomasbancrofti* sp. n., while the remaining two represent *T.* cf. *avium*. Therefore applying techniques such as culture, TEM, genetic characterization and comparison with isolates from sympatric bird species, aids in species identification.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S0031182016000329.

ACKNOWLEDGEMENTS

We thank Taronga Zoo's bird keepers for their care of the birds. Taronga Wildlife Hospital veterinary nurses and veterinarians Drs Hulst, Vinette Herrin and Tobias for assistance with sample collection and Tammy McDonogh for ongoing laboratory support. Dean Ingwersen Woodland Bird and WA Programme Manager and Regent Honeyeater recovery coordinator Birdlife Australia, Glen Johnson Senior Biodiversity Officer and Elizabeth Wemyss Project Biodiversity Officer Hume Region Regional Services Department of Environment, Land, Water & Planning (DELWP) are acknowledged for their assistance in recapturing wild and pre-released captive bred birds for blood sampling. We thank Cameron Webb (ICPMR Westmead, Medical Entomology, University of Sydney) for discussions on mosquitoes at the Zoo, Nick Clark (Griffith University) for discussion around Common Mynas and Stefan Saverimuttu for assistance with Noisy Miner sample collection at Taronga Zoo during his B.Sc. (Vet) candidature. We acknowledge the support from the facilities at the Australian

Microscopy & Microanalysis Research Facility node (Sydney Microscopy and Microanalysis) at the University of Sydney and we thank Ms. Naveena Gokoolparsadh for her expert consultations.

FINANCIAL SUPPORT

This study was in part funded through the Faculty of Veterinary Science, University of Sydney diagnostic laboratory (JS, DM, KG). Captive bred pre-release health screening and blood sampling at Taronga Zoo for the 2015 release was funded in part by the NSW Office of Environment and Heritage (LV, MS, PT). VMA is supported by the International Postgraduate Research Scholarship (IPRS) and an Australian Postgraduate Award (APA) tenable at The University of Sydney.

REFERENCES

Apanius, V. (1991). Avian trypanosomes as models of hemoflagellate evolution. *Parasitology Today* **7**, 87–90.

Baker, J. R. (1976). Biology of the trypanosomes of birds. In *Biology of the Kinetoplastida* (ed. Lumsden, W. H. R.), pp. 131–174. Academic Press, London, UK.

Bennett, G. F. (1961). On the specificity and transmission of some avian trypanosomes. *Canadian Journal of Zoology* **39**, 17–33.

Bennett, G. F. and Fallis, A. M. (1960). Blood parasites of birds Algonquin Park, Canada, and a discussion of their transmission. *Canadian Journal of Zoology* **38**, 261–273.

Bennett, G. F., Earle, R. A. and Squires-Parsons, D. (1994*a*). Trypanosomes of some sub-Saharan birds. *The Onderstepoort Journal of Veterinary Research* **61**, 263–271.

Bennett, G. F., Siikamaki, P., Ratti, O., Allander, K., Gustafsson, K. and Squires-Parsons, D. (1994*b*). Trypanosomes of some Fennoscandian birds. *Memórias do Instituto Oswaldo Cruz* **89**, 531–537.

Bennett, G. F., Squires-Parsons, D. and Poldmaa, T. (1994*c*). The species of *Haemoproteus, Leucocytozoon* and *Trypanosoma* of the Australian honeyeater family Meliphagida (Aves: Passeriformes). *Memoirs of the Queensland Museum* **37**, 13–18.

Breinl, A. (1913). Parasitic protozoa encountered in the blood of Australian native animals. Report of the Australian Institute of Tropical Medicine for 1911 (April, 1913), 30–38.

Clark, N. J., Olsson-Pons, S., Ishtiaq, F. and Clegg, S. M. (2015). Specialist enemies, generalist weapons and the potential spread of exotic pathogens: malaria parasites in a highly invasive bird. *International Journal for Parasitology* **45**, 891–899.

Cleland, J. B. and Johnston, T. H. (1910). The haematozoa of Australian birds - No. I. *Transactions and Proceedings and Report of the Royal Society of South Australia* **34**, 100–114.

Cleland, J. B. and Johnston, T. H. (1911). The haematozoa of Australian birds - No. II. *Journal of the Proceedings of the Royal Society of New South Wales* **45**, 415–444.

Crusz, H. (1984). Parasites of endemic and relic vertebrates: a biogeographical review. In *Ecology and Biogeography in Sri Lanka (Series: Monographiae Biologicae, Vol. 57)* (ed. Fernando, C. H.), pp. 321–351. Dr W. Junk Publishers, Kluwer Academic Publishing Group, Hague.

Dirie, M. F., Wallbanks, K. R., Molyneux, D. H. and McNamara, J. (1991). Comparison of *Trypanosoma grayi*-like isolates from west and east Africa. *Annals of Tropical Medicine and Parasitology* **85**, 49–52.

Driskell, A. C. and Christidis, L. (2004). Phylogeny and evolution of the Australo-Papuan honeyeaters (Passeriformes, Meliphagidae). *Molecular Phylogenetics and Evolution* **31**, 943–960.

Fleiss, J. L., Levin, B. and Paik, M. C. (2013). *Statistical Methods for Rates and Proportions*. Wiley, Hoboken, NJ.

Franklin, D. C., Menkhorst, P. W. and Robinson, J. L. (1989). Ecology of the Regent honeyeater (*Xanthomyza phrygia*). *Emu* **89**, 140–154.

Gardner, J. L., Trueman, J. W., Ebert, D., Joseph, L. and Magrath, R. D. (2010). Phylogeny and evolution of the Meliphagoidea, the largest radiation of Australasian songbirds. *Molecular Phylogenetics and Evolution* **55**, 1087–1102.

Haag, J., O'hUigin, C. and Overath, P. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology* **91**, 37–49.

Hamilton, P. B., Stevens, J. R., Gaunt, M. W., Gidley, J. and Gibson, W. C. (2004). Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. *International Journal for Parasitology* **34**, 1393–1404.

Hartigan, A., Phalen, D. N. and Šlapeta, J. (2010). Museum material reveals a frog parasite emergence after the invasion of the cane toad in Australia. *Parasites & Vectors* **3**, 50.

Hemmerter, S., Šlapeta, J. and Beebe, N. W. (2009). Resolving genetic diversity in Australasian *Culex mosquitoes*: incongruence between the mitochondrial cytochrome c oxidase I and nuclear acetylcholine esterase 2. *Molecular Phylogenetics and Evolution* **50**, 317–325.

Liu, S. C., Gillespie, J., Atchison, N. and Andrew, P. (2014). The recovery programme for the Regent honeyeater *Anthochaera phrygia*: an example of conservation collaboration in Australia. *International Zoo Yearbook* **48**, 83–91.

Lukeš, J. and Votýpka, J. (2000). *Trypanosoma avium*: novel features of the kinetoplast structure. *Experimental Parasitology* **96**, 178–181.

Mackerras, M. J. and Mackerras, I. M. (1960). The Haematozoa of Australian birds. *Australian Journal of Zoology* **8**, 226–260.

MacNally, R., Bowen, M., Howes, A., McAlpine, C. A. and Maron, M. (2012). Despotic, high-impact species and the subcontinental scale control of avian assemblage structure. *Ecology* **93**, 668–678.

McInnes, L. M., Gillett, A., Ryan, U. M., Austen, J., Campbell, R. S., Hanger, J. and Reid, S. A. (2009). *Trypanosoma irwini* n. sp (Sarcomastigophora: Trypanosomatidae) from the koala (*Phascolarctos cinereus*). *Parasitology* **136**, 875–885.

Molyneux, D. and Gordon, E. (1975). Studies on immunity with three species of avian trypanosomes. *Parasitology* **70**, 181–187.

Molyneux, D. H., Cooper, J. E. and Smith, W. J. (1983). Studies on the pathology of an avian trypanosome (*T. bouffardi*) infection in experimentally infected canaries. *Parasitology* **87**(Pt 1), 49–54.

Morin-Adeline, V., Vogelnest, L., Dhand, N. K., Shiels, M., Angus, W. and Šlapeta, J. (2011). Afternoon shedding of a new species of *Isospora* (Apicomplexa) in the endangered Regent Honeyeater (*Xanthomyza phrygia*). *Parasitology* **138**, 713–724.

O'Donoghue, P. J. and Adlard, R. D. (2000). Catalogue of protozoan parasites recorded in Australia. *Memoirs of the Queensland Museum* **45**, 1–163.

Podlipaev, A. A. (1990). Catalogue of world fauna of Trypanosomatidae (Protozoa). *Proceedings of the Zoology Institute of the USSR Academy of Science, Leningrad* **144**, 1–178.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. (2012). MrBayes 3·2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

Russell, R. C. (2012). A review of the status and significance of the species within the *Culex pipiens* group in Australia. *Journal of the American Mosquito Control Association* **28**, 24–27.

Sehgal, R. N., Jones, H. I. and Smith, T. B. (2001). Host specificity and incidence of *Trypanosoma* in some African rainforest birds: a molecular approach. *Molecular Ecology* **10**, 2319–2327.

Sehgal, R. N., Valkiūnas, G., Iezhova, T. A. and Smith, T. B. (2006). Blood parasites of chickens in Uganda and Cameroon with molecular descriptions of *Leucocytozoon schoutedeni* and *Trypanosoma gallinarum*. *The Journal of Parasitology* **92**, 1336–1343.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Thomas, R. (2009). Regent honeyeater habitat restoration project Lurg Hills, Victoria. *Ecological Management & Restoration* **19**, 84–97.

Valkiūnas, G. (2005). *Avian Malaria Parasites and other Haemosporidia*. CRC Press, Boca Raton, FL.

Valkiūnas, G., Iezhova, T. A., Carlson, J. S. and Sehgal, R. N. (2011). Two new *Trypanosoma* species from African birds, with notes on the taxonomy of avian trypanosomes. *The Journal of Parasitology* **97**, 924–930.

Votýpka, J. and Svobodová, M. (2004). *Trypanosoma avium*: experimental transmission from black flies to canaries. *Parasitology Research* **92**, 147–151.

Votýpka, J., Oborník, M., Volf, P., Svobodová, M. and Lukeš, J. (2002). *Trypanosoma avium* of raptors (Falconiformes): phylogeny and identification of vectors. *Parasitology* **125**, 253–263.

Votýpka, J., Szabová, J., Radrová, J., Zídková, L. and Svobodová, M. (2012). *Trypanosoma culicavium* sp. nov., an avian trypanosome transmitted by *Culex* mosquitoes. *International Journal of Systematic and Evolutionary Microbiology* **62**, 745–754.

Votýpka, J., d'Avila-Levy, C. M., Grellier, P., Maslov, D. A., Lukeš, J. and Yurchenko, V. (2015). New approaches to systematics of Trypanosomatidae: criteria for taxonomic (re)description. *Trends in Parasitology* **31**, 460–469.

Wallace, F. G., Camargo, E. P., McGhee, R. B. and Roitman, I. (1983). Guidelines for the description of new species of lower trypanosomatids. *The Journal of Protozoology* **30**, 308–313.

Wheeler, R. J., Gluenz, E. and Gull, K. (2013). The limits on trypanosomatid morphological diversity. *PLoS One* **8**, e79581.

Yurchenko, V., Hobza, R., Benada, O. and Lukes, J. (1999). *Trypanosoma avium*: large minicircles in the kinetoplast DNA. *Exp Parasitol* **92**, 215–218.

Zídková, L., Cepicka, I., Szabová, J. and Svobodová, M. (2012). Biodiversity of avian trypanosomes. *Infection, Genetics and Evolution* **12**, 102–112.