Occurrence and diversity of avian haemosporidia in Afrotropical landbirds

Mamohale E. Chaisi[a,*], Samuel T. Osinubi[b], Desire L. Dalton[a], Essa Suleman[a,c]

[a] Department of Research and Scientific Services, National Zoological Garden, South African National Biodiversity Institute (SANBI), 232 Boom Street, Pretoria, 0001, South Africa
[b] FitzPatrick Institute of African Ornithology, University of Cape Town, Private Bag X3, Rondebosch, 7701, Cape Town, South Africa
[c] Council for Scientific and Industrial Research (CSIR), P.O. Box 395, Pretoria, 0001, South Africa

ARTICLE INFO

ABSTRACT

Avian haemosporidian infections are widespread and can result in the decline of wild bird populations or in some cases contribute to extinction of species. We determined the prevalence and genetic diversity of avian haemosporidia in 93 samples from 22 landbird species from South Africa (N = 76) and West Africa (N = 17), of which six are intra-African migrants and one is a Palearctic migrant. The samples were analysed for the presence of avian haemosporidian DNA using real-time quantitative PCR (qPCR) and nested PCR assays targeting specific mitochondrial genes of these parasites. The cytochrome *b* (*cyt*b) gene was sequenced for all samples that tested positive and phylogenetic analysis was conducted in order to determine the relationship of the new sequences with previously published sequences from the MalAvi database. The overall prevalence of avian haemosporidiosis was 68.82% (95% CI: 56.4%–78.87%) and 82.80% (95% CI: 65.68%–86.11%) as determined by qPCR and nested PCR respectively. Eighteen (19.36%; 95% CI; 10.78%–29.97%) samples had mixed infections. Infection prevalence of all haemosporidian spp. were significantly higher (p < 0.05) in samples from West Africa. Forty-six mitochondrial sequences obtained from 14 avian species grouped into three distinct clusters of *Haemoproteus* (36), *Leucocytozoon* (8) and *Plasmodium* (2). These represent eight published and nine new *cyt*b lineages. The most common lineage was *Haemoproteus* sp. (VIMWE1) which was identified in two bird species from West Africa and seven bird species from South Africa. This study adds to our knowledge of host-parasite relationships of avian haemosporidia of Afrotropical birds.

1. Introduction

Over 200 species of avian haemosporidian species (spp.) have been described (Hellgren et al., 2004; Valkiūnas, 2005; Braga et al., 2011; Yoshimura et al., 2014). Infection by *Haemoproteus* and *Plasmodium* spp. can cause haemoproteosis and avian malaria respectively, while infection by *Leucocytozoon* spp. can result in leucocytozoonosis. Haemosporidian infections can range from asymptomatic to severe and potentially fatal disease depending on the parasite lineage and bird species (Palinauskas et al., 2008, 2009; 2011, 2016). Immunologically naïve birds and juveniles are highly susceptible to infection (Atkinson, 2005; Smith and Ramey, 2015), resulting in mortality at their first exposure (Bueno et al., 2010; Jia et al., 2018). Pathogenic haemosporidian species have been associated with the decline and death of native and endangered avian species in Hawaii (Atkinson and Samuel, 2010), New Zealand (Alley et al., 2008; Niebuhr et al., 2016), Australia (Cannell et al., 2013) and the Galapagos islands (Levin et al., 2013) and are therefore a conservation threat, especially in captivity where animal

densities are high (Alley et al., 2008; Grilo et al., 2016). Birds that survive the acute phase become chronically infected and may act as reservoirs of infection to vectors and susceptible birds. Monitoring parasite infections in wild bird populations assists in assessing the risk of transmission of infections to domestic and endangered birds (Valkiūnas, 2005).

Studies on African rainforest birds have indicated a vast diversity of haemosporidia in these hosts (Richard et al., 2002; Valkiūnas et al., 2008; Beadell et al., 2009; Iezhova et al., 2011; Ishtiaq et al., 2012; Njabo et al., 2012; Hellgren et al., 2015; Lutz et al., 2015). However, knowledge of their distribution and their vectors in some parts of Sub-Saharan Africa is lacking (Loiseau et al., 2012; Njabo et al., 2012). In South Africa, research on avian haemosporidia has mostly focused on sea birds, which experience high infection rates as well as a huge diversity of avian haemosporidia (Okanga et al., 2014). A study at the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) found that African Penguins (*Spheniscus demersus*) presented for rehabilitation at the centre were highly susceptible to

---

infection and usually acquired infection during rehabilitation (Botes et al., 2017). Strict vector control measures resulted in the decline of new malaria infections in these endangered birds.

Migratory birds usually harbour high infections and biologically diverse avian haemosporidian species (Smith and Ramey, 2015) and can act as a source of infection to non-migratory and captive birds (Bueno et al., 2010; Yoshimura et al., 2014). Their role in the transmission and introduction of pathogens to new environments is well documented (Ogden et al., 2008; Levin et al., 2013; Yoshimura et al., 2014). Previously, the detection of avian haemosporidia relied mainly on the microscopic examination of blood smears (Valkiūnas, 2005), however advances in molecular biology have provided more rapid, specific and sensitive methods for the detection of these parasites by use of the polymerase chain reaction (PCR; Waldenström et al., 2004; Iezhova et al., 2011; Bell et al., 2015). Studies of avian haemosporidia using PCR based assays have increased our knowledge and understanding of their vectors, prevalence, diversity and distribution in different habitats and climatic zones, phylogenetic relationships, and host-parasite interactions (Atkinson, 2005; Loiseau et al., 2012; Cannell et al., 2013; Hellgren et al., 2015; Sehgal, 2015; Palinauskas et al., 2016; Carbó-Ramírez et al., 2017). The aim of this study was to determine the occurrence, diversity and phylogenetic relationships of avian haemosporidia in free-ranging birds from South Africa and West Africa using PCR based assays and sequencing of the cytochrome *b* (*cyt*b) gene.

2. Materials and methods

2.1. Samples and DNA extraction

Blood samples were collected from ninety-three healthy adult and sub-adult birds, representing six orders, 15 families and 22 species, of which 6 are intra-African migrants, one is a Palearctic migrant and 15 are resident in the study sites (Table 1). The samples were collected in South Africa (N = 76) and West Africa (N = 17) using varying numbers and lengths of mist nets and spring traps baited with superworms (*Zophobas moria*). The traps were deployed during morning (06h00-10h00) and evening (15h00-18h00) sessions. In South Africa, the samples were collected from three localities (Mogalakwena, Venetia and Mokopane) in the Limpopo Province between December 2015 and January 2016. In West Africa, samples were collected from two countries (Nigeria and Ghana) between May and June 2016. In Nigeria, sampling was done at the A.P. Leventis Ornithological Research Institute (APLORI), Jos. In Ghana, samples were collected at sites in Accra and Damongo. Captured birds were ringed to ensure individuality of samples, measured, sexed, aged, tissue samples collected and immediately released after sampling. Blood samples were collected using the brachial venipuncture method with 27-gauge needles and 100-μl capillary tubes, and the blood samples stored in lysis buffer (Seutin et al., 1991). All biological materials collected were stored at the Biobank of the National Zoological Garden, South African National Biodiversity Institute (NZG, SANBI).

2.2. qPCR and nested PCR assays

DNA was extracted from 100 μl of storage mix of blood and lysis buffer using the Quick-gDNA™ Mini Prep Extraction kit (Zymo Research, Inqaba Biotec, South Africa) according to the manufacturer's instructions and eluted with 30 μl of elution buffer. The concentration of DNA was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and the extracted DNA was then diluted to a concentration of 20 ng/μl with molecular grade ddH$_2$O and stored at −20 °C prior to further analysis. A synthetic DNA fragment (gBlocks®, Integrated DNA Technologies (IDT), Whitehead

Table 1
Occurrence of avian haemosporidian infections in different bird species as detected by the qPCR and nested PCR assays.

Order	Family	Host species	Total	Nested PCR results			qPCR results	
				Leuco[a]	Plas/Haem[a]	% infected	Number[b]	% infected
South Africa			**76**					
Bucerotiformes	Upupidae	African Hoopoe (*Upupa africana*)[c]	1	0	1	100	1	100
Columbiformes	Columbidae	Cape Turtle Dove (*Streptopelia capicola*)	1	0	1	100	0	0
Coraciiformes	Alcedinidae	African Pygmy Kingfisher (*Ispidina picta*)[c]	8	0	3	35.7	3	35.7
		Brown-hooded Kingfisher (*Halcyon albiventris*)[e]	1	1	1	100	1	100
	Dacelonidae	Woodland Kingfisher (*Halcyon senegalensis*)[e, c]	26	4	20	79.92	16	61.54
Cuculiformes	Cuculidae	Diederik Cuckoo (*Chrysococcyx caprius*)[e, c]	21	3	17	80.95	13	61.91
		Klaas' Cuckoo (*Chrysococcyx klaas*)[e, c]	2	1	2	100	2	100
		Red-chested Cuckoo (*Cuculus solitarius*)[c]	4	0	4	100	2	50
Passeriformes	Laniidae	Red-backed Shrike (*Lanius collurio*)[d]	1	0	1	100	1	100
	Leiothrichidae	Arrow-marked Babbler (*Turdoides jardineii*)	1	0	1	100	1	100
	Cuculiformes	Southern Black Tit (*Parus niger*)	1	0	1	100	1	100
		Red-headed Weaver (*Anaplectes rubriceps*)	1	0	1	100	1	100
		Southern Masked Weaver (*Ploceus velatus*)[e]	2	1	2	100	2	100
	Passeriformes	Village Weaver (*Ploceus cucullatus*)	1	0	0	0	0	0
	Pycnonotidae	Yellow-bellied Greenbul (*Chlorocichla flaviventris*)[e]	1	1	1	100	1	100
	Turdidae	Kurrichane Thrush (*Turdus libonyana*)[e]	1	1	1	100	1	100
Piciformes	Indicatoridae	Lesser Honeyguide (*Indicator minor*)	2	0	0	0	1	50
	Lybiidae	Crested Barbet (*Trachyphonus vaillantii*)[e]	1	1	1	100	1	100
West Africa			**17**					
Coraciiformes	Dacelonidae	Woodland Kingfisher (*Halcyon senegalensis*)[e]	3	1	3	100	3	100
Cuculiformes	Cuculidae	DiederikCuckoo (*Chrysococcyx caprius*)	7	3	7	100	6	85.71
	Meropidae	Red-throated Bee-eater (*Merops bulocki*)	1	1	0	100	1	100
Passeriformes	Muscicapidae	Snowy-crowned Robin-chat (*Cossypha niveicapilla*)[e]	1	1	1	100	1	100
	Pycnonotidae	Common Bulbul (*Pycnonotus barbatus*)	2	2	0	100	2	100
	Turdidae	African Thrush (*Turdus pelios*)[e]	3	3	3	100	3	100

[a] *Leuco - Leucocytozoon; Plas/Haem – Plasmodium and/or Haemoproteus*.
[b] The assay does not differentiate between haemosporidian genera.
[c] Intra-African migrants.
[d] Palearctic migrant.
[e] Mixed infections of *Leucocytozoon* and *Haemoproteus/Plasmodium* infections.

Scientific, South Africa) of the *Plasmodium relictum* sequence (GenBank accession # NC012426) spanning a 1394 bp region of ribosomal DNA (rDNA) and *cytochrome b* (*cytb*) gene of the mitochondrial genome was used as a positive control, and molecular grade water as a negative control. The dry pellet of the synthetic DNA fragment was resuspended in 100 μl of TE buffer to a final concentration of 10 ng/μl. To determine the sensitivity of the qPCR assay, seven ten-fold dilutions (6.8×10^5–0.68 gene copies/μl) of the *Plasmodium* control DNA fragment were analysed in triplicate in two separate plate runs. Gene copy number (copies/μl) was calculated as follows: (amount (ng/μl) $\times 6.022 \times 10^{23}$)/(length (bp) $\times 1 \times 10^9 \times 650$) (Ke et al., 2006). The dilutions and field samples were analysed for the presence of haemosporidian DNA using a recently described qPCR assay (Bell et al., 2015). The qPCR reaction mix consisted of 7.5 μl PowerUp SYBR Green mix (Thermo Fisher Scientific, South Africa), 0.4 μM of forward (R330F) and reverse (R480RL) primers (Bell et al., 2015), 3 μl (60 ng) of sample DNA and ddH$_2$O to a total volume of 15 μl and was analysed using the QuantStudio 12K Flex Real-Time PCR instrument (Applied Biosystems, California, USA). Cycling conditions were as follows: UDG activation at 50 ℃ for 2 min; initial denaturation at 95 ℃ for 2 min; 40 cycles of amplification at 95 ℃ for 15 s; 53 ℃ for 15 s and 72 ℃ for 1 min, and a final extension at 72 ℃ for 1 min, followed by melt curve analysis using the default settings of the instrument. The qPCR assay targets a 182 bp region of the mitochondrial rDNA and amplifies a conserved region common to all three haemosporidian genera using a single set of primers (Bell et al., 2015). Baseline, quantitative cycle (Cq), melting temperature (Tm) and gene copy number (GCN) of the test samples and controls were determined using the quantitative analysis mode of the QuantStudio 12K Flex software. The efficiency (E) of the assay was calculated from the slope of the standard curve.

The nested PCR assay (Bensch et al., 2000; Hellgren et al., 2004) was used to amplify the *cytb* gene of the avian haemosporidia. The primary PCR amplifies DNA from all three genera and was performed using 2 μl (40 ng) sample DNA, 12.5 μl DreamTaq master mix (Thermo Fisher Scientific), 4 μM of primers HaemNF1 and HaemNR3 and molecular grade ddH$_2$O to a final volume of 25 μl. The secondary nested PCR reactions were performed using 1 μl of the primary reaction to amplify a fragment of approximately 480bp of *Leucocytozoon* spp. with primers HaemFL and HaemR2L or *Plasmodium/Haemoproteus* spp. with primers HaemF and HaemR2. Synthetic DNA (gBlocks®) of *P. relictum* (as indicated above) and *Leucocytozoon fringillinarun* (GenBank accession # FJ168564) were used as positive controls. PCR cycling conditions were as follows: initial denaturation at 95 ℃ for 5 min, 20 cycles (primary PCR) and 35 cycles (secondary nested PCRs) of denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s, extension at 72 ℃ for 45 s, followed by a final extension at 72 ℃ for 10 min. The resulting amplicons were visualised on a 1.5% agarose gel stained using theSYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

2.3. Sequence and phylogenetics analyses

The PCR products (20 μl) from all positive samples were purified using 1 μl Exonuclease I and 2 μl FastAp Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) prior to cycle sequencing. The sequencing reaction of 10 μl contained 0.7 μl BigDye™ (Thermo Fisher Scientific), 2.55 μl sequencing buffer, 10 μM of each nested secondary PCR primer (HAEMF and HAEMR2) for *Plasmodium* and *Haemoproteus* spp., or HAEMFL and HAEMR2L for *Leucocytozoon* spp. separately, 5 μl of PCR product and 0.75 μl molecular grade ddH$_2$O. Cycle sequencing was conducted at 94 ℃ for 2 min; 40 cycles of amplification at 85 ℃ for 10 s; 53 ℃ for 10 s and 60 ℃ for 2.5 min. The sequencing reactions were cleaned-up using 55 μl SAM solution per sample of the BigDye XTerminator kit (Thermo Fisher Scientific) prior to analysis on the ABI 3130xl Automated DNA Sequencer (Applied Biosystems, Foster City, California). Forward and reverse reads were assembled and edited using the CLC Main Workbench programme (CLC Bio, Boston, MA, USA).

Mixed sequences, as indicated by contigs with double peaks, were considered as co-infections (Lutz et al., 2015) and were excluded from further analysis. A search for homologous sequences was done from the MalAvi database (Bensch et al., 2009; http://mbio-serv2.mbioekol.lu.se/Malavi/) using the Basic Local Alignment Search Tool (BLAST). Mitochondrial haplotypes i.e. sequences differing by one or more bases (< 100% identity) from known parasite lineages were described as unique lineages (Hellgren et al., 2004).

The sequences were submitted to GenBank (Accession numbers MH492266 – MH492311). The new sequences were aligned with homologous sequences from MalAvi using the CLC Main Workbench programme7 (http://www.clcbio.com). Phylogenetic relationships were inferred from 49 *cytb* sequences (470 bp) using MEGA7 (Kumar et al., 2016) for the Neighbor-Joining tree, with 1000 bootstrap replicates, and Bayesian inference (BI) using MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001) as implemented in Geneious version R11.1.5 (www.geneious.com). Posterior probabilities for the BI were determined from 100,000 generations, sampled every 500 generations. The General Time Reversible with 5 gamma distributions (GTR + G) model was determined by MEGA7 as the best fit model and was used in both analyses. The trees were edited using MEGA7.

2.4. Statistical analysis

Microsoft Excel (Microsoft Office 2010) and SYSTAT version 13 (https://systatsoftware.com/products/systat/) software were used for data analyses. The prevalence of avian haemosporidian infections was estimated at a significance level of 5% ($p < 0.05$) and 95% confidence interval (CI). Resampling was conducted with 1000 bootstrap samples. The chi-squared (χ^2) test was used to determine the association between the qPCR and nested PCR results, and the level of agreement between the results of the two assays was determined by the Cohen's Kappa coefficient (*k*) (Landis and Koch, 1977) at a 95% CI.

3. Results

3.1. qPCR assay

The qPCR assay that we employed in this study detects all three avian haemosporidian genera in a single reaction (Bell et al., 2015) and was used as an initial screening assay. The efficiency of the qPCR assay, as determined from the slope of the standard curve, was 100% (Fig. 1). The linear detection range of the assay is 6.8×10^5–0.68 gene copies/μl. The lowest detection limit of the assay is 2.04 copies of the *cytb* gene (0.68 gene copies/3 μl DNA). The average Cq range (17.03–38.18) and mean melting temperature (Tm = 78.88 ± 0.09℃) of the serial dilutions were obtained from two runs (six values per dilution). Positive samples had Cq values ≤ 38.5 so this value was determined as the cut-off. There was no amplification in the negative control. The assay detected avian haemoparasite DNA in 64 (68.82%; 95% CI: 56.4%–78.87%) of 93 samples; Tm 78.52℃ - 80.64℃ (Mean 79.20 ˚C ± 0.36℃). Parasitaemia, as indicated by the Cq values and GCN, was generally low with 68.75% of samples with Cq > 30 (≥130GCN); and 31.25% with Cq < 30 (GCN ≥ 370) (Fig. 1). The highest level of infection (GCN = 2 900 690) was from an African Pygmy Kingfisher (*Ispidina picta*) that was captured in Mokopane, South Africa.

3.2. Nested PCR assay

The sensitivity of the nested PCR assay was assessed by analysing serial dilutions (6.8×10^5–0.68 gene copies/μl) of the *P. relictum* control as was done with the qPCR assay. Amplicons of approximately 480 bp were obtained from the nested PCR results as expected. The lowest linear range of detection by the nested PCR assay was 13.6 gene copies per reaction (6.8 gene copies/2 μl DNA); there was no amplification in
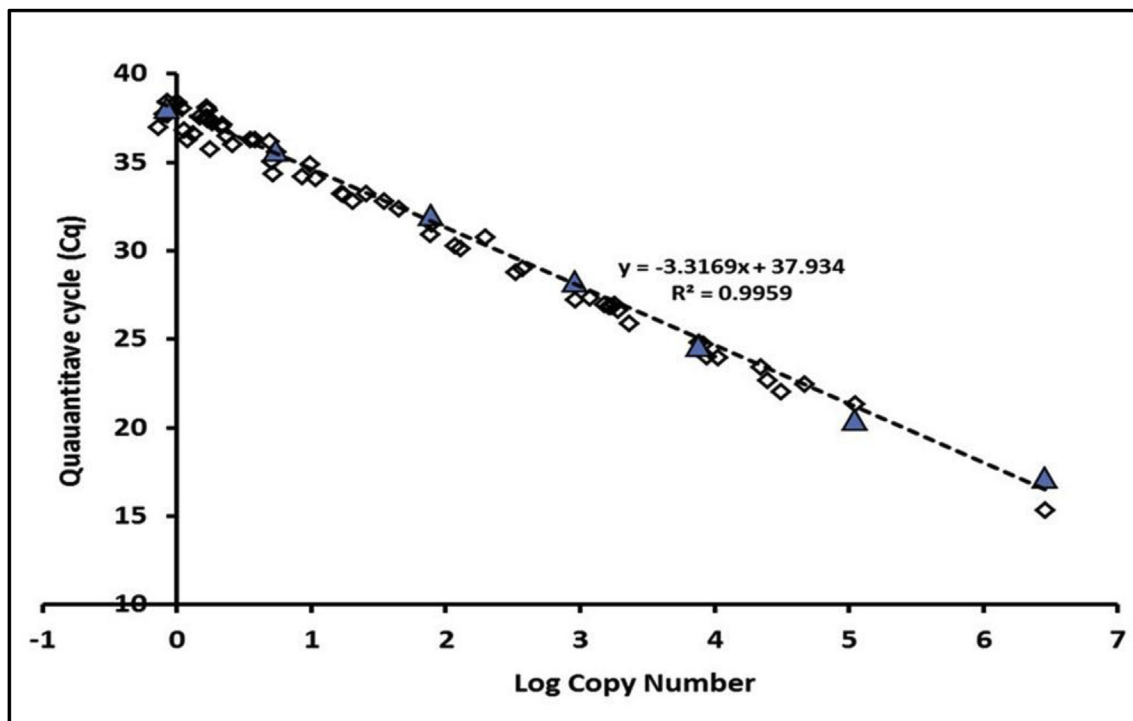
Fig. 1. Standard curve of 10-fold serial dilutions (6.8 × 10⁵–0.68 gene copies/ul) of genomic DNA (gBlocks) of *Plasmodium relictum* (triangles) and field samples (diamonds).

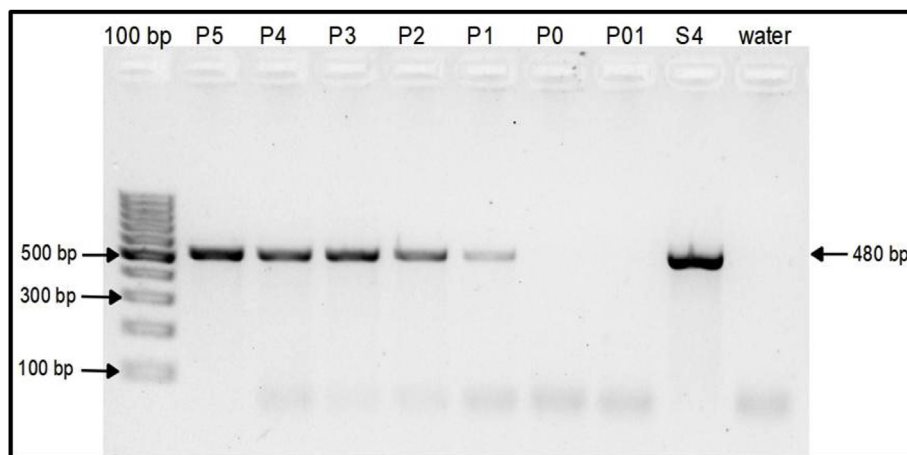**Fig. 2.** Gel electrophoresis image of nested PCR of ten-fold serial dilutions of *Plasmodium. relictum* positive control amplicons (P5 – P01; 6.8 × 10⁵–0.68 gene copies/μl DNA).
S4 – Sample from an African Pygmy Kingfisher; water - negative control.

the negative control (Fig. 2). A total of 77 (82.80%: 95% CI; 65.68%–86.11%) birds were infected with at least one haemosporidian species. All bird species, with the exception of the Cape Turtle Dove (*Streptopelia capicola*) and Village Weaver (*Ploceus cucullatus*), were infected by at least one haemosporidian species (Table 1).

Infection rates between the birds from South Africa and West Africa were significantly different (Table 2). Overall, infections were 63.13% and 94.12% by the qPCR assay, and 78.95% and 100% by the nested PCR in birds from South Africa (n = 76) and West Africa (n = 17) respectively (Table 2). *Leucocytozoon* spp. infections were detected in 25.81% of all samples, and were significantly higher (p < 0.05) in West African birds (64.71%) than in birds from South Africa (17.11%). *Plasmodium/Haemoproteus* spp. occurred in 77.49% of all samples. Similarly, higher infections of *Plasmodium/Haemoproteus* were detected in birds from West Africa (82.35%) than in South African birds (76.32%;

Table 2). Mixed infections of *Leucocytozoon* and *Plasmodium/Haemoproteus* occurred in 19.32% (95% CI; 10.78%–29.97%) of all samples and were significantly different (p < 0.05) in samples from South African and West Africa (Table 2). These were identified in eight bird species: Brown-hooded Kingfisher (*Halcyon albiventris*), Woodland Kingfisher (*Halcyon senegalensis*), Diederik Cuckoo (*Chrysococcyx caprius*) Klaas Cuckoo (*Chrysococcyx klaas*), Snowy-crowned Robin Chat (*Cossyphaniveicapilla*), Yellow-bellied Greenbul (*Chlorocichla flaviventris*); Kurrichane Thrush (*Turdus libonyana*) and Crested Barbet (*Trachyphonus vaillantii*; Table 1).

3.3. Comparison of the qPCR and nested PCR assays

Sixty-four and 77 samples were detected as positive by qPCR and nested PCR, respectively. This suggests that the nested PCR assay was

Table 2

The prevalence of avian haemosporidian infections in migratory birds from South Africa and West Africa.

Infection	Number (%) positive via qPCR[a]			Number (%) positive via nested PCR			Cohen's kappa (95% CI)
	South Africa (n = 76)	West Africa (n = 17)	TOTAL (n = 93)	South Africa (n = 76)	West Africa (n = 17)	TOTAL (n = 93)	
Avian haemosporidia	48 (63.16%)	16 (94.12%)	64 (68.82%)[d]	60 (78.95%)	17 (100%)	77 (82.80%)[d]	0.115 (−0.086 to 0.316)[c]
Leucocytozoon				13 (17.11%)	11 (64.71%)	24 (25.81%)[d]	
Haemoproteus/Plasmodium				58 (76.32%)	14 (82.35%)	72 (77.49%)[d]	
Mixed Infections[b]				10 (13.16%)	8 (47.06%)	18 (19.36%)[d]	

[a] The assay does not differentiate between the different genera.

[b] *Haemoproteus and/or Plasmodium*, and *Leucocytozoon*. The prevalence of avian haemosporidia was significantly higher in samples from West Africa (p < 0.05).

[c] The strength of agreement between the two assays is considered to be poor.

[d] CI; Confidence interval. Cohen's kappa coefficient (*k*) (ranges from −1 to +1) measures the strength of agreement between the qPCR and nested PCR assays.

more sensitive in detecting avian haemosporidian parasites although the difference was not statistically different ($\chi^2 = 1.422$; 1df; p = 0.233) (Table 2). There was agreement between the results of the two assays in 62 samples (66.76%; 55 positive; 7 negative), a relatively high proportion (22; 23.7%) of the samples were positive by nested PCR but negative by qPCR, and nine samples tested positive by qPCR and negative by the nested PCR assay. The strength of agreement between the two assays was considered to be poor according to Cohen's Kappa coefficient (*k* = 0.115; CI 95% −0.860–0.316; Table 2).

3.4. Haemosporidian lineage diversity and phylogenetics

We obtained a total of 46 haemosporidian sequences from 44 samples and 14 host species (Fig. 3). Thirty-six sequences were from bird samples collected in South Africa; 10 from West African birds. We identified 17 lineages (Fig. 3), nine of which are new lineages. Bird species, MalAvi BLAST results, lineages and GenBank Accession numbers are listed in Supplementary Table 1. Pairwise sequence comparisons indicated that there are 1–3 nucleotide differences between sequences of the same clade. The greatest number of lineages was

identified from the Woodland Kingfisher (*Halcyon senegalensis*) (7 lineages) and Diederik Cuckoo (*Chrysococcyx caprius*) (6 lineages), the other hosts displayed only one or two lineages (Fig. 3). The sequences were homologous (94%–100%) to published sequences from the MalAvi database and they grouped into distinct, highly supported, clades of *Haemoproteus*, *Leucocytozoon* and *Plasmodium* spp. (Fig. 4). The topologies of the Neighbor-joining tree and Bayesian trees were observed to be similar. The Neighbor-joining tree is shown (Fig. 4). Some lineages have alternative names in the MalAvi database, we used the originally described lineage names to avoid confusion.

Nine *Haemoproteus* lineages were identified from 11 bird species from South Africa and West Africa (Appendix 1); two of these (VIMWE1 and CHRKLA01) were identified in samples from both localities. Lineage VIMWE1 was the most commonly identified and was obtained from eight host species (Supplementary Table 1). The other lineages that we identified are HALMAL01 from two Woodland Kingfishers from South Africa, ALCLEU02 from the African Pygmy Kingfisher and BUL2 (*Heamoproteus sanguinis*) from the Diederik Cuckoo from Nigeria (Fig. 3). The new lineages CHRCAP01, CHRKLA01, HALSEN01, HALSEN02 and HALSEN03 were identified from different host species
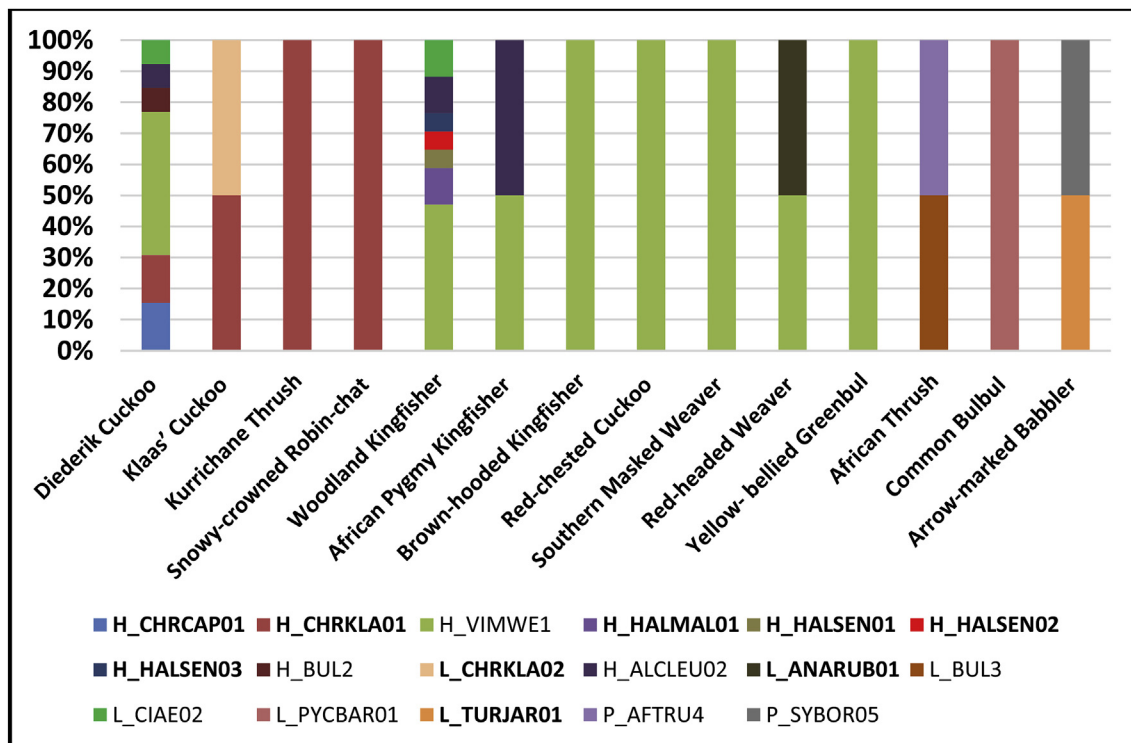

Fig. 3. Haemosporidian lineage diversity (%) in Afrotropical birds.

H_ *Haemoproteus*; L_ *Leucocytozoon*; P_ *Plasmodium*. Unique lineages from this study are in bold.
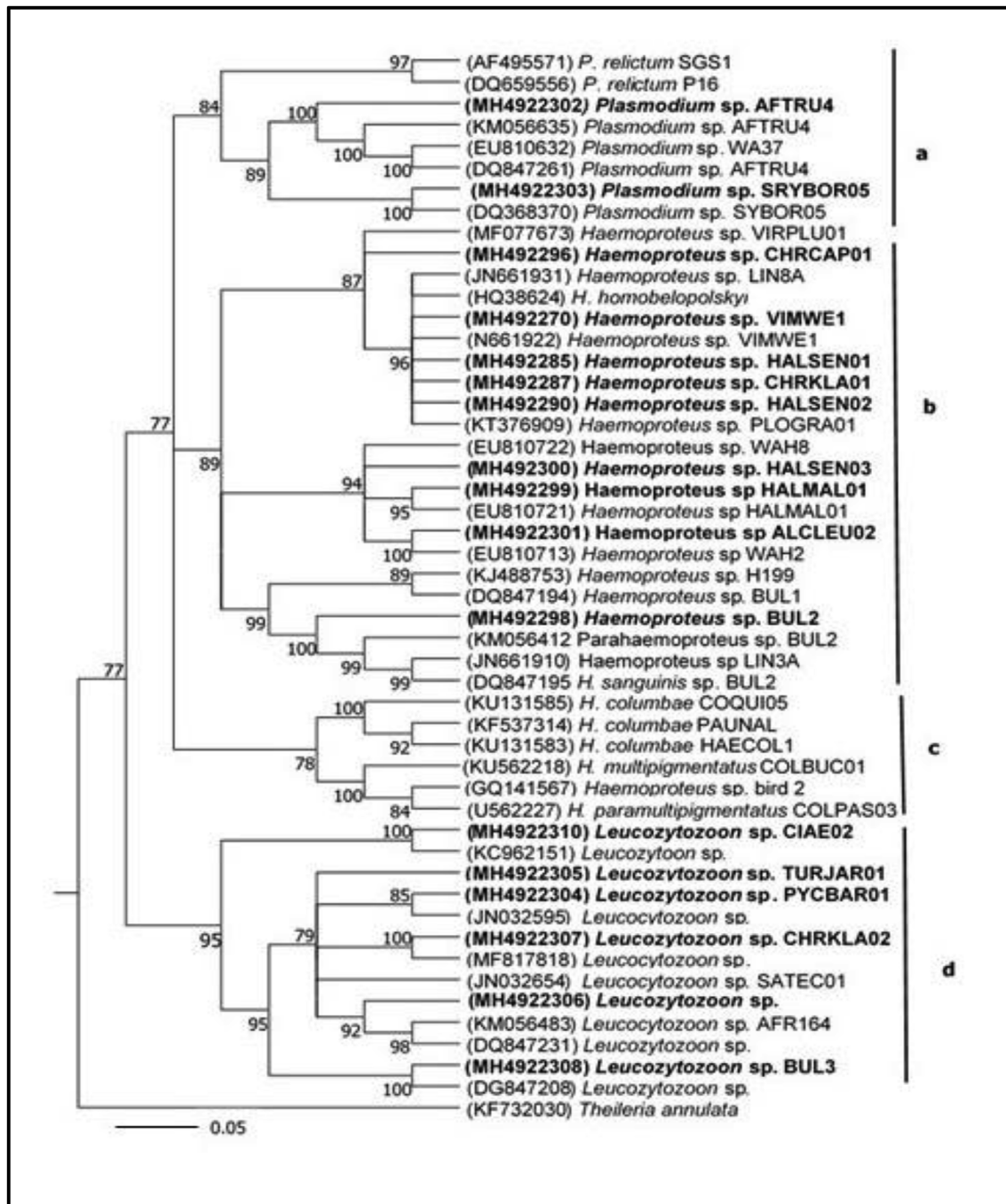
Fig. 4. The phylogenetic relationships of 49 *cytochrome b* sequences of avian haemosporidian species, *Plasmodium* (**a**), *Heamoproteus*: *Parahaemoproteus* subgenera (**b**), *Haemoproteus* subgenera (**c**), and *Leucozytozoon* (**d**). Bootstrap values (> 75%) are indicated next to the branch nodes. GenBank accession numbers are indicated in parenthesis, followed by the species name and MalAvi lineage. Representative lineages obtained from this study are in bold. The *cytochrome b* sequence of *Theileria annulata* is used as the outgroup.

from South Africa and West Africa (Appendix 1).

Six *Leucocytozoon* lineages were obtained from eight samples; four of these are described for the first time in this study (Fig. 3). Two Woodland Kingfishers and a Diederik Cuckoo from South Africa were infected by lineage CIAE02. The new lineages: CHRLAR02 (South Africa), ANARUB01 (South Africa), PYCBAR01 (Ghana, West Africa) and TURJAR01 (South Africa) were identified from samples obtained from the Klaas' Cuckoo (*Chrysococcyx klaas*), Red-headed Weaver (*Anaplectes rubriceps*), Common Bulbul (*Pycnonotus barbatus*) and the Arrow-marked Babbler (*Turdoides jardineii*), respectively.

We obtained only two *Plasmodium* sequences from the African

Thrush (*Turdus pelios*) (Ghana) and Arrow-marked Babbler from Ghana and South Africa, respectively. The former was infected by lineage AFTU4, and the latter was infected by lineage SYBOR5 which was previously described from the Garden Warbler (*Sylvia borin*) in Europe (Pérez-Tris et al., 2007).

4. Discussion

4.1. Overall prevalence

Since parasitaemia may be low in chronic infections, the

identification of avian haemosporidia using multiple screening methods increases the probability of detecting these infections (Lutz et al., 2015). Therefore, we used a qPCR assay and two nested assays to detect haemosporidian infections in intra-migratory birds originating from South Africa and West Africa (Nigeria and Uganda). The results of the two assays indicated that the birds sampled harboured high rates of infections (68.82%–82.80%) of avian haemosporidia, with significantly higher prevalence of *Haemoproteus/Plasmodium* spp. and *Leucocytozoon* spp. in samples from West Africa. These results are consistent with those from previous studies on avian haemoparasites in West and East Africa as haemosporidian infections in tropical African birds are generally high and diverse (Valkiūnas et al., 2008; Beadell et al., 2009; Iezhova et al., 2011; Lutz et al., 2015). However, we did not analyse the samples by microscopy to determine the presence of gametocytes in the host blood cells which would indicate that positively infected birds could transmit the infections. Avian haemosporidia species can switch hosts, evolving into new lineages, however, such infections are abortive and cannot be transmitted to a vector as the parasite does not develop fully in the non-adaptive host due incompatibilities between the parasite and the host (Valkiūnas, 2011; Palinauskas et al., 2016; Valkiūnas and Iezhova, 2017). Molecular-based methods can overlook such infections as the PCR can detect sporozoites or DNA from extra-cellular merozoites even when gametocytes are absent in blood smears (Levin et al., 2013).

Studies carried out in other parts of the world indicated that the prevalence of avian haemosporidia differs according to the species sampled, methodology, geographical location and seasonality, making it difficult to compare results obtained from these studies (Valkiūnas et al., 2003; Levin et al., 2013; Yoshimura et al., 2014; Smith and Ramey, 2015; Schmid et al., 2017). In this study, samples were collected during the peak breeding season for the intra-African migrants from both localities and therefore climatic conditions and activity of the insect vectors are similar. The highest level of infection (GCN = 2 900 690) was from an African Pygmy Kingfisher that was captured in Mokopane, South Africa. Sequencing results indicated that this bird was infected with *Haemoproteus* lineage ALCEU02, previously identified from the Kingfisher in Gabon (Beadell et al., 2009). Migratory birds are often exposed to higher parasite diversity and can aid in transmission of pathogens and their vectors along their migratory routes (Rappole et al., 2000; Waldenstrom et al., 2002; Jourdain et al., 2007; Levin et al., 2013).

Although parasitaemia in wild birds is usually low (Palinauskas et al., 2016), they may act as asymptomatic hosts and reservoirs of infections to susceptible hosts. Some avian species are not exposed to these species in their natural environments due to the absence of vectors and are therefore highly susceptible to infections. Furthermore, low parasitaemia is not always a measure of bird health as infection may have detrimental effects on a bird's fitness by interrupting the production of blood cells in the bone marrow and other organs (Palinauskas et al., 2016). Additionally, mist-nests and other traditional bird capture methods generally capture birds that are at the chronic stage of infection (with low parasitaemia) and may under-sample acutely (usually inactive) infected birds (Valkiūnas, 2005). Njabo et al. (2012) suggested that alternative sampling methods should be used in order to determine the true prevalence of these infections in bird populations. Mixed species infections, as observed in our study, are common in wild birds and are generally considered more virulent than single infections (Jarvi et al., 2013; Schmid et al., 2017).

4.2. Comparison of PCR assays

The qPCR assay of Bell et al. (2015) was developed to target the conserved rRNA gene of the mitochondrial DNA of avian haemosporidianparasites. It offers a rapid, reliable and cost-effective method for the detection of avian haemoparasite infections. We compared the performance of this assay to that of the nested PCR (Hellgren et al.,

2004) in detecting these infections. Although the difference in the detection of the malaria species by the two assays (77 by nested PCR; 64 by qPCR) was not statistically significant (χ^2 = 1.422; 1df; p = 0.233), there was only a slight agreement between the results of the two assays as the nested PCR assay detected approximately 20% more infections than the qPCR assay. These results indicate that there could be sequence variations in the target region of the qPCR assay in the samples tested in this study which would compromise the specificity of the assay due to mismatch in parasite DNA and primers, resulting in false negatives. The shift in melting temperature (Tm) in *Haemoproteus* positive samples (Tm = 79.30ºC), as opposed to samples that were positive for *Plasmodium* (Tm = 78.88 ºC) or *Leucocytozoon* (Tm = 78.91 ºC) indicates that there is some variation in the qPCR target area, which may further hinder the assay in detecting some genotypes/lineages. However, the extent of the variation can only be determined by sequencing the rRNA gene in field samples where this discordance was observed. This was outside the scope of this study. Additionally, there could be proportionally more amplified parasite DNA in the second round of the nested PCR than in the qPCR assay, thereby increasing the chances of detecting infections. In the study by Bell et al. (2015), there was no significant difference in the detection of avian haemosporidian parasites by qPCR and nested PCR protocols. However, in another study (Ishtiaq et al., 2017) the qPCR assay was more sensitive than the nested PCR assay in detecting infections in samples from India, but there was no difference in the ability of both assays in detecting infections from Swedish birds, indicating that geographical variants may determine the specificity of the assay.

4.3. Lineage biodiversity

Genetic diversity of haemosporidian parasites in the wild birds was high, with a total of 17 lineages of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* spp. from 14 of the 21 bird species that were sampled (Fig. 3). Lutz et al. (2015) indicated that with sufficient sampling, the number of malaria parasite lineages within a geographic area should approximate the number of host species. Our results may therefore have underestimated the degree of biodiversity in the study area, partly due the fact that we could not obtain sequences from all the positive samples. The greatest diversity of avian haemoparasite species has been reported from Afrotropical birds, where 149 lineages of *Plasmodium* and *Haemoproteus* spp. were found from 152 bird species (Lutz et al., 2015). Some of the lineages we identified are identical to those that were previously identified from wild birds in other parts of Africa and elsewhere in the world (Beadell et al., 2009; Iezhova et al., 2011; Ishtiaq et al., 2012; Fourcade et al., 2014; Lutz et al., 2015). The greatest number of lineages was obtained from the Woodland Kingfisher (7) and Diederik Cuckoo (6) as these species had the highest numbers of samples. Infections with more than one haemosporidian lineage were identified from two samples from South Africa. One sample was infected with *Leucocytotoon* lineage CHRKLA02 and *Haemoproteus* lineage CHRKLA01, and the other sample had a mixed infection of *Leucocytozoon* lineage TURJAR01 and *Plasmodium* lineage SYBOR05 (Appendix 1). These results were in concordance with the nested PCR results which indicated the presence of mixed infections.

The small sample sizes for most of the hosts limits us from determining host specificity of the obtained lineages; however, some trends in host specificity are apparent. Lineage AFTRU4 has been identified from members of the thrush family in Malawi (Lutz et al., 2015) and Gabon (Beadell et al., 2009), we also identified this lineage from a sample from the African Thrush (*Turdus pelios*) from Ghana. The new lineage, CHRCAP01 was identified from infections of two Diederik cuckoo samples from South Africa. However, infections by the two most common lineages (VIMWE1 and CHRKLA01) occurred in different bird species from the two geographical localities (Supplementary Table 1). Lineage VIMWE1 was previously identified from the Lesser Masked Weaver (*Ploceus intermedius*) in Botswana (Ishtiaq et al., 2012).

Lineages ALCLEU02 and HALMAL01 were previously identified from the White-bellied Kingfisher (*Alcedo leucogaster*) and Blue-breasted Kingfisher (*Halcyon malimbica*) in Gabon (Beadell et al., 2009). Lineage BUL2 (*Haemoproteus sanguinis*) is a common lineage of Bulbuls (family Pycnonotidae) and it is the only lineage that we identified to species level. It was initially described from India (Chakravarty and Kar, 1945) and has since been found in infections of Bulbul species from Hong Kong, the Philippines, Indonesia, Malaysia, Thailand, India, Ghana, Zaire, Uganda, Kenya, Tanzania, South Africa, Aldabra, the Comoro Islands (Bennett et al., 1982; Rahal et al., 1987) and Malawi (Lutz et al., 2015). We identified this lineage from a Diederik Cuckoo from Nigeria.

Six *Leucocytozoon* lineages were obtained from eight samples; four of these are described for the first time in this study (Fig. 3). Two Woodland Kingfishers and a Diederik Cuckoo from South Africa were infected by lineage CIAE02 which was previously described in raptor birds and other bird species in Europe (Krone et al., 2008; Silva-Iturriza et al., 2012; Ciloglu et al., 2016). According to the MalAvi database, this lineage has been identified from 16 host species. We identified lineage BUL3 from the African Thrush (*Turdus pelios*) that was captured in Nigeria. This lineage was previously described from the Common Bulbul (*Pycnonotus barbatus*) in Nigeria (Hellgren et al., 2007). PCR analysis indicated that the two samples from the Common Bulbul from West Africa were infected with *Leucozytozoon* spp. and we obtained a novel lineage, PYCBAR01, from one of the samples. The other new lineages: CHRLAR02 (South Africa), ANARUB01 (South Africa), and TURJAR01 (South Africa) were identified from samples obtained from the Klaas' Cuckoo (*Chrysococcyx klaas*), Red-headed Weaver (*Anaplectes rubriceps*), and the Arrow-marked Babbler (*Turdoides jardineii*), respectively.

Two *Plasmodium* sequences were obtained from the African Thrush (Ghana) and Arrow-marked Babbler (South Africa). The former was infected by lineage AFTU4, previously identified from the Kurrichane Thrush in Malawi (Lutz et al., 2015). The latter was infected by lineage SYBOR5 which was previously described from the Garden Warbler (*Sylvia borin*) in Europe (Pérez-Tris et al., 2007).

There is currently no consensus on the naming of new/unique lineages based on *cyt*b sequences (Schmid et al., 2017), and different authors have used different approaches in naming these lineages. We used single nucleotide differences to define new lineages as is the case in the MalAvi database (Bensch et al., 2000; Hellgren, 2005; Chagas et al., 2017). However, Levin et al. (2013) used a sequence divergence of more than two base pairs to define new lineages.

Plasmodium lineages are considered as generalists, while *Leucozytozoon* and *Haemoproteus* lineages are generally host specific (Beadell et al., 2009). However, we identified *Haemoproteus* spp. more frequently, and in a wider range of bird species than *Plasmodium* spp. Although this result might indicate the active movement of biting midges in the study area, biodiversity in the other species may have been underestimated by using conventional PCR methods and direct sequencing as these methods do not detect mixed infections (Bernotiene et al., 2016). The detection of mixed species and lineage infections in conservation projects is important as these infections are usually associated with virulence (Bernotiene et al., 2016). A comparison of the sensitivities of five commonly used PCR assays for detecting avian malaria infections by these authors indicated that all the assays underestimated mixed infections, and that although the PCR assay described by Bensch et al. (2000), primers (HaemNFI/NR3 and HAEMF/R2), was the most sensitive in detecting mixed infections of *Haemoproteus* spp., primers 3760F/4292Rw2 described by Beadell et al. (2004) were more sensitive in detecting mixed infections of *Plasmodium* spp. This could explain why we obtained a greater number of *Haemoproteus* sequences than those of *Plasmodium* using primers HaemNFI/NR3 and HAEMF/R2. Bernotiene et al. (2016) further indicated that most (90%) of the mixed haemosporidian infections were detected using three primer sets; apicoplast (Martinsen et al., 2008), and *cyt*b (3760F/4292Rw2 and HaemNFI/NR3, HAEMF/R2) in parallel and

therefore recommended that at least three primer sets should be used in parallel for detecting mixed infections of *Haemoproteus* and *Plasmodium* spp., especially in wildlife where there is a huge diversity of these species. PCR suppression and competition of primers have also been reported in assays involving multiple infections of closely related species and could have also contributed to the observed results. The use of different primer sets for the primary PCR of *Plasmodium* and *Haemoproteus* spp. in other studies (Waldenstrom et al., 2004; Ishtiaq et al., 2017; Schmid et al., 2017) resulted in a significant increase in the number of infections detected compared to the assay of Bensch et al. (2000). Cloning and sequencing is also more effective than direct sequencing of PCRproductsand has been used to determine avian haemosporidian lineages in mixed infections (Pérez-Tris and Bensch, 2005). However, these methods are time-consuming and might not be practical in surveillance studies. High resolution melt (HRM) analyses was also found to be more sensitive than the nested PCR in detecting *Plasmodium* infections in mosquitoes (Njabo et al., 2012). More recently, next generation sequencing (NGS) was used to determine sequence variations within the pathogenic *P. relictum* GRW4 strain lineage (Jarvi et al., 2013). Although NGS is expensive, it allows for increased depth of coverage and is therefore more informative than direct sequencing or cloning (Jarvi et al., 2013).

In conclusion, our study adds to the knowledge of host-parasite relationships of avian haemosporidia in free ranging birds in sub-Saharan Africa, and further indicates that these infections are common and diverse. Infected migratory birds may act as reservoirs for these haemoparasites which may further result in infection of captive and endangered birds threatening their conservation. Future studies should determine the possible role of these birds in the spread and/or persistence of these infections across Africa.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2018.12.002.

References

Alley, M.R., Fairley, R.A., Martin, D.G., Howe, L., Atkinson, T., 2008. An outbreak of avian malaria in captive yellowheads/mohua (*Mohoua ochrocephala*). N. Z. Vet. J. 56, 247–251.

Atkinson, C.T., 2005. Ecology and Diagnosis of Introduced Avian Malaria in Hawaiian Forest Birds. USGS Numbered Ser. 1–4.

Atkinson, C.T., Samuel, M.D., 2010. Avian malaria *Plasmodium relictum* in native Hawaiian forest birds: epizootiology and demographic impacts on àapapane *Himatione sanguinea*. J. Avian Biol. 41, 357–366.

Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., Atkinson, C.T., Fleischer, R.C., 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Mol. Ecol. 13, 3829–3844.

Beadell, J.S., Covas, R., Gebhard, C., Ishtiaq, F., Melo, M., Schmidt, B.K., Perkins, S.L., Graves, G.R., Fleischer, R.C., 2009. Host associations and evolutionary relationships of avian blood parasites from West Africa. Int. J. Parasitol. 39, 257–266.

Bell, J.A., Weckstein, J.D., Fecchio, A., Tkach, V.V., 2015. A new real-time PCR protocol for detection of avian haemosporidians. Parasites Vectors 8, 383.

Bennett, G.F., Whiteway, M., Woodworth-Lynas, C., 1982. Host-parasite catalogue of avian Haematozoa. Occas. Pap. Biol. 5, 1–243.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. R. Soc. B Biol. Sci. 267, 1583–1589.

Bensch, S., Hellgren, O., Pérez-Tris, J., 2009. MalAvi: a public database of malaria

parasites and related haemosporidians in avian hosts based on mitochondrial *cytochrome b* lineages. Mol. Ecol. Resour. 9, 1353–1358.

Bernotiene, R., Palinauskas, V., Iezhova, T., Murauskaite, D., Valkiūnas, G., 2016. Avian haemosporidian parasites (Haemosporida): a comparative analysis of different polymerase chain reaction assays in detection of mixed infections. Exp. Parasitol. 163, 31–37.

Botes, A., Thiart, H., Parsons, N.J., Bellstedt, D.U., 2017. Conservation implications of avian malaria exposure for African penguins during rehabilitation. South Afr. J. Sci. 113 (7/8).

Braga, E.M., Silveira, P., Belo, N.O., Valkiūnas, G., 2011. Recent advances in the study of avian malaria: an overview with an emphasis on the distribution of *Plasmodium* spp in Brazil. Mem. Inst. Oswaldo Cruz 106, 3–11.

Bueno, M.G., Lopez, R.P., de Menezes, R.M., Costa-Nascimento Mde, J., Lima, G.F., Araújo, R.A., Guida, F.J., Kirchgatter, K., 2010. Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. Vet. Parasitol. 173, 123–127.

Cannell, B.L., Krasnec, K.V., Campbell, K., Jones, H.I., Miller, R.D., Stephens, N., 2013. The pathology and pathogenicity of a novel *Haemoproteus* spp. Infection in wild Little Penguins (*Eudyptula minor*). Vet. Parasitol. 197, 74–84.

Carbó-Ramírez, P., Zuria, I., Schaefer, H.M., Santiago-Alarcon, D., 2017. Avian haemosporidians at three environmentally contrasting urban greenspaces. J. Urban Econ. 3, 1–11.

Chagas, C.R.F., Valkiūnas, G., de Oliveira Guimarães, L., Monteiro, E.F., Guida, F.J.V., Simões, R.F., Rodrigues, P.T., de Albuquerque Luna, E.J., Kirchgatter, K., 2017. Diversity and distribution of avian malaria and related haemosporidian parasites in captive birds from a Brazilian megalopolis. Malar. J. 16, 83.

Chakravarty, M., Kar, A.B., 1945. Studies on *haemosporidia* from Indian birds. Series II. Proc. Indian Acad. Sci. 22B, 63–69.

Ciloglu, A., Yildirim, A., Duzlu, O., Onder, Z., Dogan, Z., Inci, A., 2016. Investigation of avian haemosporidian parasites from raptor birds in Turkey, with molecular characterisation and microscopic confirmation. Folia Parasitol. 63, 023.

Fourcade, Y., Keišs, O., Richardson, D.S., Secondi, J., 2014. Continental-scale patterns of pathogen prevalence: a case study on the corncrake. Evol. Appl. 7, 1043–1055.

Grilo, M.L., Vanstreels, R.E.T., Wallace, R., García-Párraga, D., Braga, É.M., Chitty, J., Catão-Dias, J.L., Madeira de Carvalho, L.M., 2016. Malaria in penguins – current perceptions. Avian Pathol. 9457, 1–51.

Hellgren, O., Waldenström, J., Bensch, S., 2004. A new PRCassay for simultaneous studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* from avian blood. J. Parasitol. 90, 797–802.

Hellgren, O., 2005. The occurrence of haemosporidian parasites in the Fennoscandian bluethroat (Luscinia svecica) population. J. Ornithol. 146, 55–60.

Hellgren, O., Waldenstrom, J., Perez-Tris, J., Szoll, E., Si, O., Hasselquist, D., Krizanauskiene, A., Ottosson, U., Bensch, S., 2007. Detecting shifts of transmission areas in avian blood parasites – a phylogenetic approach. Mol. Ecol. 16, 1281–1290.

Hellgren, O., Atkinson, C.T., Bensch, S., Albayrak, T., Dimitrov, D., Ewen, J.G., Kim, K.S., Lima, M.R., Martin, L., Palinauskas, V., Ricklefs, R., Sehgal, R.N.M., Valkiūnas, G., Tsuda, Y., Marzal, A., 2015. Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity. Ecography 38, 842–850.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Iezhova, T.A., Dodge, M., Sehgal, R.N.M., Smith, T.B., Valkiūnas, G., 2011. New avian *Haemoproteus* species (Haemosporida: Haemoproteidae) from African birds, with a critique of the use of host taxonomic information in hemoproteid classification. J. Parasitol. 97, 682–694.

Ishtiaq, F., Beadell, J.S., Warren, B.H., Fleischer, R.C., 2012. Diversity and distribution of avian haematozoan parasites in the western Indian Ocean region: a molecular survey. Parasitology 139, 221–231.

Ishtiaq, F., Rao, M., Huang, X., Bensch, S., 2017. Estimating prevalence of avian haemosporidians in natural populations : a comparative study on screening protocols. Parasit. Vectors 10, 127.

Jarvi, S.I., Farias, M.E., Lapointe, D.A., Belcaid, M., Atkinson, C.T., 2013. Next-generation sequencing reveals cryptic mtDNA diversity of *Plasmodium relictum* in the Hawaiian Islands. Parasitology 140, 1741–1750.

Jia, T., Huang, X., Zhang, C., 2018. Malaria parasites and related haemosporidians cause mortality in cranes: A study on the parasites diversity, prevalence and distribution in Beijing Zoo. Malar J. 17, 234.

Jourdain, E., Gauthier-Clerc, M., Bicout, D.J., Sabatier, P., 2007. Bird migration routes and risk for pathogen dispersion into western Mediterranean wetlands. Emerg. Infect. Dis. 13, 365–372.

Ke, G.M., Cheng, H.L., Ke, L.Y., Ji, W.T., Chulu, J.L.C., Liao, M.H., Chang, T.J., Liu, H.J., 2006. Development of a quantitative Light Cycler real-time RT-PCR for detection of avian reovirus. J. Virol. Methods 133, 6–13.

Krone, O., Waldenström, J., Valkiūnas, G., Lessow, O., Müller, K., Iezhova, T.A., Fickel, J., Bensch, S., 2008. Haemosporidian blood parasites in European birds of prey and owls. J. Parasitol. 94, 709–715.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159.

Levin, I.I., Zwiers, P., Deem, S.L., Geest, E.A., Higashiguchi, J.M., Iezhova, T.A., Jiménez-Uzcátegui, G., Kim, D.H., Morton, J.P., Perlut, N.G., Renfrew, R.B., Sari, E.H.R., Valkiunas, G., Parker, P.G., 2013. Multiple lineages of avian malaria parasites

(*Plasmodium*) in the Galapagos islands and evidence for arrival via migratory birds. Conserv. Biol. 27, 1366–1377.

Loiseau, C., Harrigan, R.J., Robert, A., Bowie, R.C.K., Thomassen, H.A., Smith, T.B., Sehgal, R.N.M., 2012. Host and habitat specialization of avian malaria in Africa. Mol. Ecol. 21, 431–441.

Lutz, H.L., Hochachka, W.M., Engel, J.I., Bell, J.A., Tkach, V.V., Bates, J.M., Hackett, S.J., Weckstein, J.D., 2015. Parasite prevalence corresponds to host life history in a diverse assemblage of afrotropical birds and haemosporidian parasites. PLoS One 10, 1–24.

Martinsen, E.S., Perkins, S.L., Schall, J.J., 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol. Phylogenet. Evol. 47, 261–273.

Niebuhr, C.N., Poulin, R., Tompkins, D.M., 2016. Is avian malaria playing a role in native bird declines in New Zealand? Testing hypotheses along an elevational gradient. PLoS One 11, 1–16.

Njabo, K.Y., Cornel, A.J., Bonneaud, C., Toffelmier, E., Valki, G., Russell, A.F., Smith, T.B., 2012. Parasite associations in a central African rainforest. Mol. Ecol. 20, 1049–1061.

Ogden, N.H., Lindsay, L.R., Hanincova, K., Barker, I.K., Bigras-Poulin, M., Charron, D.F., Heagy, A., Francis, C.M., O'Callaghan, C.J., Schwartz, I., Thompson, R.A., 2008. Role of migratory birds in introduction and range expansion of I*xodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Appl. Environ. Microbiol. 74, 1780–1790.

Okanga, S., Cumming, G.S., Hockey, P.A.R., Nupen, L., Peters, J.L., 2014. Host specificity and co-speciation in avian haemosporidia in the Western Cape, South Africa. PLoS One 9.

Palinauskas, V., Valkiūnas, G., Bolshakov, C.V., Bensch, S., 2008. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp. Parasitol. 120, 372–380.

Palinauskas, V., Valkiūnas, G., Križanauskiene, A., Bensch, S., Bolshakov, C.V., 2009. *Plasmodium relictum* (lineage P-SGS1): further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone™. Exp. Parasitol. 123, 134–139.

Palinauskas, V., Valkiūnas, G., Bolshakov, C.V., Bensch, S., 2011. *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): the effects of theco-infection on experimentally infected passerine birds. Exp. Parasitol. 27, 527–533.

Palinauskas, V., Žiegytė, R., Iezhova, T.A., Ilgūnas, M., Bernotienė, R., Valkiūnas, G., 2016. Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. Int. J. Parasitol. 46, 697–707.

Pérez-Tris, J., Bensch, S., 2005. Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. Parasitology 131, 15–23.

Pérez-Tris, J., Hellgren, O., Križanauskiene, A., Waldenström, J., Secondi, J., Bonneaud, C., Fjeldså, J., Hasselquist, D., Bensch, S., 2007. Within-host speciation of malaria parasites. PLoS One 2, e235.

Rahal, E.M., Bishop, M.A., Bennett, G.F., 1987. Avian Haemoproteidae. 25. The haemoproteids of the avian family Pycnonotidae (bulbuls). Can. J. Zool. 65, 322–328.

Rappole, J.H., Derrickson, S.R., Hubálek, Z., 2000. Migratory birds and spread of west nile virus in the western Hemisphere. Emerg. Infect. Dis. 6, 319–328.

Richard, F.A., Sehgal, R.N.M., Jones, H.I., Smith, T.B., 2002. A comparative analysis of PCR-based detection methods for avian malaria. J. Parasitol. 88, 819–822.

Schmid, S., Fachet, K., Dinkel, A., Mackenstedt, U., Woog, F., 2017. Carrion crows (*Corvus corone*) of southwest Germany: important hosts for haemosporidian parasites. Malar. J. 16, 369.

Sehgal, R.N.M., 2015. Manifold habitat effects on the prevalence and diversity of avian blood parasites. Int. J. Parasitol. Parasites Wildl. 4, 421–430.

Seutin, G., White, B., Boag, P., 1991. Preservation of avian blood and tissue samples for DNA analyses. Can. J. Zool. Can. Zool. 69, 82–90.

Silva-Iturriza, A., Ketmaier, V., Tiedemann, R., 2012. Prevalence of avian haemosporidian parasites in the central Phillippine islands. Parasitol. Int. 61, 650–657.

Smith, M.M., Ramey, A.M., 2015. Prevalence and genetic diversity of haematozoa in South American waterfowl and evidence for intercontinental redistribution of parasites by migratory birds. Int. J. Parasitol. Parasites Wildl. 4, 22–28.

Valkiūnas, G., Iezhova, T.A., Shapoval, A.P., 2003. High prevalence of blood parasites in hawfinch *Coccothraustes coccothraustes*. J. Nat. Hist. 37, 2647–2652.

Valkiūnas, G., 2005. Avian Malaria Parasites and Other Haemosporidia. CRC, Boca Raton, Florida.

Valkiūnas, G., 2011. Haemosporidian vector research:marriage of molecular and microscopical approaches is essential. Mol. Ecol. 20, 3084–3086.

Valkiūnas, G., Iezhova, T.A., Loiseau, C., Chasar, A., Smith, T.B., Sehgal, R.N.M., 2008. New species of haemosporidian parasites (Haemosporida) from African rainforest birds, with remarks on their classification. Parasitol. Res. 103, 1213–1228.

Valkiūnas, G., Iezhova, T.A., 2017. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malar. J. 16, 101.

Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D., Ottosson, U., 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol. Ecol. 11, 1545–1554.

Waldenström, A.J., Bensch, S., Hasselquist, D., Östman, Ö., 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J. Parasitol. 90, 191–194.

Yoshimura, A., Koketsu, M., Bando, H., Saiki, E., Suzuki, M., Watanabe, Y., Kanuka, H., Fukumoto, S., 2014. Phylogenetic comparison of avian haemosporidian parasites from resident and migratory birds in Northern Japan. J. Wildl. Dis. 50, 235–242.