

Blood Parasites of Vangas and Other Corvoidea on Madagascar

Regina Magaña Vázquez¹, Friederike Woog², Anke Dinkel¹, Ute Mackenstedt¹ and Sandrine Musa^{1,*}¹ Department of Parasitology, University of Hohenheim, Emil-Wolff-Str. 34, 70599 Stuttgart, Germany² State Museum of Natural History Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany

* Correspondence: sandrine.musa@uni-hohenheim.de

Abstract: Madagascar hosts a great diversity of bird species. This study focuses on the description of the diversity and prevalence of blood parasites (Haemosporida, trypanosomes and filarioid nematodes) in 131 blood samples of 14 species of Corvoidea, namely vangas (Vangidae), *Coracina cinerea* (Campephagidae), *Dicrurus forficatus* (Dicruridae) and *Terpsiphone mutata* (Monarchidae) found in primary rainforests on Madagascar. Blood parasites were detected using both molecular and microscopic methods. Multiplex PCR was used to detect mixed haemosporidian infections and nested PCR was used to describe a 479 bp fragment of the haemosporidian cytochrome b (*cytb*) gene. Furthermore, a 770 bp SSU rRNA fragment of trypanosomes, and, for microfilariae, a 690 bp fragment of 28S rRNA, as well as a 770 bp fragment of 28S rRNA, were amplified for identification using nested PCRs. Phylogenetic analyses were carried out for all sequences obtained from all blood parasite taxa. Over half of the samples (54.2%; n = 71) were infected with Haemosporida, whereas only 21.4% (n = 28) were infected with *Trypanosoma* and 5.3% (n = 7) contained filarioid nematode DNA. Fourteen of 56 blood smears contained some of the above-mentioned parasite taxa. The results corroborate the great diversity of blood parasites in the different bird species studied, especially in vangas. Vangas had the greatest diversity of parasites found, as well as the highest number of multiple infections, which may be due to their morphological diversity and resulting habitat use. Fifteen haemosporidian lineages, seven *Trypanosoma* and five filarioid nematode isolates were newly discovered in the avian species studied, particularly in the vangas. Members of the other Corvoidea families on Madagascar showed a lower susceptibility for avian haemosporidian parasites than vangas, which could be attributed to possible resistance against those parasites. The study confirmed the host specificity of some Haemosporida and microfilariae; however, it demonstrated that this was not the case for *Trypanosoma*.

Keywords: Corvoidea; Vangidae; Haemosporida; *Trypanosoma*; microfilariae

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1. Introduction

The island of Madagascar is classified as an important biodiversity hotspot [1]. This is not due to a particularly high number of species, but to an extremely high proportion of endemic species. For example, Madagascar harbors a total of 292 bird species, of which 108 are reported to be strictly endemic to this island [2]. The most prominent bird family endemic to Madagascar, the Vangidae, are an excellent example of adaptive radiation. Soon after colonizing the island, a single ancestral species showed explosive bursts of diversification, evolving striking differences in bill morphology that allowed exploitation of the extremely varied climatic conditions and habitats of the island [3,4]. Today, 15 genera of primarily insectivorous Vangidae exist of which 12 genera are monospecific [2]. Besides the vangas, most species of the superfamily Corvoidea present in Madagascar are also endemic. *Terpsiphone* paradise flycatchers (Monarchidae) are a globally widespread and highly speciose genus of passerines occurring over most of sub-Saharan Africa, southern and eastern Asia, the Philippines, and the western Indian Ocean islands [5]. Several of the *Terpsiphone* species are migratory. Three non-migratory species are endemic to the western Indian Ocean and are split into eight subspecies found amongst the different

islands, with the Madagascar paradise flycatcher *Terpsiphone mutata mutata* being endemic to Madagascar [2]. The drongos (Dicruridae) include only the genus *Dicrurus*, comprising 21 insectivorous species [6]. All members of the family are morphologically highly homogenous being of similar size and showing a glossy black coloration and a more or less forked tail [7]. The Malagasy region hosts four species, each endemic to a certain island. One subspecies of the crested drongo *Dicrurus forficatus forficatus* is strictly endemic to Madagascar [2]. The Campephagidae represent an Old-World clade of tropical birds with more than 70 species [8]. The genus *Coracina* is the most diversified (50 species) with many species being endemic to small and remote islands. In contrast to drongos, *Coracina* species are rather disparate morphologically [9]. Three species occur in the Malagasy region of which only the ashy cuckoo-shrike *Coracina cinerea* is endemic to Madagascar [2]. The vangas, Madagascar paradise flycatcher, crested drongo and the ashy cuckoo-shrike can form mixed-species flocks [10].

Studies on blood parasites of the above mentioned Corvoidea species are rare. Morphological studies [11–14] have emphasized the identification of all kinds of blood parasites infecting birds (Haemosporida, trypanosomes and filarioid nematodes), whereas more recent molecular studies have focused mainly on the detection and description of haemosporidian parasites [15,16]. The order Haemosporida includes vector-borne protozoan parasites of the four genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Fallisia*. Being the ancestors of malaria parasites, including human malaria, and because of their importance as disease-causing agents, avian Haemosporida are of great interest [17]. Previous studies identified an enormous number of haemosporidian lineages worldwide from a great variety of bird species. Data from over 4704 unique genetic lineages from 2182 bird species have already been collected in the MalAvi database (as of July 2022; [18]). Madagascar seems to be a biodiversity hotspot for *Plasmodium* and *Haemoproteus* species [16].

Trypanosoma is the most abundant and important genus of kinetoplasts [19]. These protozoan blood parasites infect different vertebrate hosts, where they occupy the bloodstream or an intracellular environment, and are transmitted by a wide variety of blood-sucking arthropods. Avian trypanosomes are not vertebrate-host specific; rather, strains successfully develop in numerous species of distant avian hosts [20]. In contrast to their mammalian relatives, avian trypanosomes are, in most cases, harmless to their hosts and remain understudied [21]—there are only seven well-characterized species from birds so far for which molecular and morphological data are available [19]. Filarioid nematodes are highly specialized parasites of vertebrates with a worldwide distribution. Adults infect tissue and tissue spaces, whereas larval stages can be found in the peripheral blood as circulating microfilariae [22]. Different blood-sucking insects are intermediate hosts of parasitic nematodes and enable transmission [23]. Although microfilariae are quite easy to detect microscopically, identification is challenging due to the similarities in their morphology. Of the approximately 160 avian filarioid species described to date, DNA sequences of only six species are currently available, which makes molecular identification usually impossible [22].

This study provides new insights into the diversity of these blood parasites present in vangas and other endemic Corvoidea bird species on Madagascar. Molecular data on blood parasites, such as the Haemosporida, trypanosomes and microfilariae, provide evidence for inter- and intra-specific co-occurrences.

2. Materials and Methods

Blood samples were collected in the Maromizaha rainforest located in the eastern part of Madagascar (18°58'8" S, 48°27'48" E), 30 km from Moramanga city, at an altitude between 943–1213 m. Birds were caught with mist nets in the months September–January (2003–2007, 2010, 2012, 2014, 2016 and 2018), with the majority of samples taken in November and December, coinciding with the breeding season of many bird species in the study area [24]. A blood sample was taken by puncturing the brachial vein. The protocol was approved by the Direction de la Préservation de la Biodiversité, Antananarivo, Madagascar. Blood

was immediately stored in lysis buffer [25], and, if possible, one blood smear per bird was prepared before the bird was released back into the wild. A total of 131 bird blood samples of different Corvoidea were collected: *Coracina cinerea* (Campechagidae, n = 4), *Dicrurus forficatus* (Dicruridae, n = 8), *Terpsiphone mutata* (Monarchidae, n = 40), and different species of the bird family Vangidae (n = 79).

Blood smears (n = 56) were prepared in the years 2007, 2012, 2014, 2016 and 2018, dried on site and fixed with 99% methanol for 10 min in the field. Using the Hemacolor® staining set (Merck KGaA, Darmstadt, Germany) the blood smears were stained with Giemsa following the manufacturer's protocol. Every slide was examined using an AxioImager M2 (Carl Zeiss AG, Oberkochen, Germany). At first, slides were screened at 400× magnification for 10 min to quickly detect larger parasites (*Leucocytozoon* spp., trypanosomes, and filarioid nematodes) and then 20 min under high magnification (×100 oil-immersion objective, ×10 ocular) to detect smaller ones (*Plasmodium* and *Haemoproteus* spp.). Pictures were taken and edited with Zen software (Carl Zeiss AG, Oberkochen, Germany).

Total DNA extraction of blood samples was performed using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. DNA concentration and purity were quantified using a NanoDrop N50 UV-Vis spectrophotometer (Implen GmbH, Munich, Germany) and stored at −20 °C until further use.

Molecular parasite detection was performed using different PCR setups. In each test run a positive control as well as a negative control (nuclease-free water) was included.

Screening for haemosporidian parasites was performed using a multiplex PCR approach [26] with specific primer pairs for simultaneous DNA amplification of *Plasmodium* (PMF/PMR), *Haemoproteus* (HMF/HMR) and *Leucocytozoon* (LMF/LMR). The reactions were set up in a total volume of 10 µL containing 5 µL of 2× Multiplex PCR Master-Mix (Quiagen, Hilden, Germany), 0.2 µL of each primer (10 µM) and 3.8 µL of DNA template. If the DNA concentration was higher than 10 ng/µL, the DNA template was diluted with nuclease-free water. Cycling conditions were performed as described by Ciloglu et al. [26]. Amplification products (10 µL) of the multiplex PCR were mixed with GelRed™ stain (BIOTREND, Köln, Germany) and electrophoretically resolved after 45 min at 90 V in 2% agarose gels. The different parasite genera apparent in the samples were determined by identifying the size of the resulting amplification fragments (*Plasmodium*: 377–379 bp; *Haemoproteus* 525–533 bp; *Leucocytozoon* 218 bp).

For identification purposes, all samples were additionally screened using a nested PCR targeting a 479 bp region of the cytochrome b gene (*cytb*) of the haemosporidian parasites [27,28]. PCR reactions of the first PCR were carried out in a total volume of 25 µL, containing 2.5 µL GeneAmp™ 10X PCR Buffer II (Applied Biosystems, Carlsbad, CA, USA), 2 µL MgCl₂ (25 mM), 1 µL of each primer (HaemNF/HaemNR3; 10 mM), 0.5 µL of each dNTP (10 µmol), 0.125 µL AmpliTaq™ DNA polymerase (5 U/µL; Applied Biosystems, Carlsbad, CA, USA), 5 µL template DNA (10–100 ng/µL) and 12.875 µL nuclease-free water. The reaction mixture of the nested PCRs consisted of 5 µL GeneAmp™ 10X PCR Buffer II (Applied Biosystems, Carlsbad, CA, USA), 4 µL MgCl₂ (25 mM), 2 µL of each primer (HaemF/HaemR2 for *Plasmodium*/*Haemoproteus* detection and HaemFL/HaemR2L for *Leucocytozoon* detection; 10 mM), 1 µL of each dNTP (10 µmol), 0.25 µL AmpliTaq™ DNA polymerase (5 U/µL; Applied Biosystems, Carlsbad, CA, USA), 2 µL amplification product of the initial PCR and 33.75 µL nuclease-free water in a total volume of 50 µL. Cycling conditions of both PCRs were performed as described by Hellgren et al. [27]. Amplification products (5 µL) of the nested PCR were also mixed with GelRed™ stain and then visualized on a 1.5% agarose gel after 20 min at 90 V.

Presence of *Trypanosoma* DNA was detected by nested PCR targeting a 770 bp SSU rRNA fragment [20]. The first set of primers was Tryp763 and Tryp1016 and the second set was Tryp99 and Tryp957. Reaction mixtures were equal to the nested PCR used for haemosporidian detection. Cycling conditions of both PCRs were performed as described by Valkiūnas et al. [20]. Amplification products (5 µL) of the nested PCR were also mixed with GelRed™ stain and then visualized on a 1.5% agarose gel after 20 min at 90 V.

The molecular detection of microfilariae was not possible using already published PCR protocols [29,30]. Probably due to low concentrations of DNA, one-step PCRs were not sufficiently sensitive. Using Geneious v. 2021.1.1 (<https://www.geneious.com>), primers for specific nested PCRs were developed. Screening for microfilariae was performed using a nested PCR assay targeting a 28S rRNA fragment. Using the primers 28SNemF1 (3'—GAGTGAAAAGGAAAAAGCCC—5') and 28SNemR1 (3'—ATCCGTGTTTCAAGACGGG—5') for the first PCR, an approximately 790 bp fragment could be amplified. The nested PCR amplified an approximately 690 bp fragment of 28S rRNA nested within the first fragment, using the primer pair 28SNemF2 (3'—ATGGAAATGTAGCGTATAGG—5') and 28SNemR2 (3'—GGTGATTAACGTTTCACATCG—5'). PCR reaction of the first PCR was carried out in a total volume of 25 µL, containing 2.5 µL GeneAmp™ 10X PCR Buffer II, 2 µL MgCl₂ (25 mM), 1 µL of each primer (10 mM), 0.5 µL of each dNTP (10 µmol), 0.125 µL AmpliTaq™ DNA polymerase, 5 µL template DNA (10–100 ng/µL) and 12.875 µL nuclease-free water. The cycling profile consisted of denaturation at 94 °C for 5 min; followed by 94 °C/30 s, 51 °C/30 s, 72 °C/45 s for 25 cycles; and a final extension at 72 °C for 7 min. The reaction mixture of the nested PCR consisted of 5 µL GeneAmp™ 10X PCR Buffer II, 4 µL MgCl₂ (25 mM), 2 µL of each primer (10 mM), 1 µL of each dNTP (10 µmol), 0.25 µL AmpliTaq™ DNA polymerase, 2 µL amplification product of the initial PCR and 33.75 µL nuclease-free water in a total volume of 50 µL. The cycling profile consisted of denaturation at 94 °C for 5 min; followed by 94 °C/30 s, 49 °C/30 s, 72 °C/45 s for 35 cycles; and a final extension at 72 °C for 7 min. Amplification products (5 µL) were mixed with GelRed™ stain and then visualized on a 1.5% agarose gel after 20 min at 90 V.

A second nested PCR, targeting a fragment of the *cox1* gene, was developed and used for samples where DNA amplification of the microfilarial 28S rRNA fragment was successful. Using the primers COINemF1 (3'—TATAATTCTGTTCTTACTATGCATGG—5') and COINemR1 (3'—GGAATAGCAATAATGATAGTAGCAGC—5') for the first PCR, an approximately 730 bp fragment was amplified. The nested PCR amplified an approximately 650 bp fragment of *cox1* nested within the first fragment, using the primer pair COINemF2 (3'—CCTATTTTGATTGGTGGTTTTGG—5') and COINemR2 (3'—CTAAAATAAGTACG—AGTATCAATATC—5'). PCR reactions were carried out as for 28S rRNA-nested PCR. The cycling profile of the first PCR consisted of denaturation at 94 °C for 5 min; followed by 94 °C/30 s, 55 °C/30 s, 72 °C/45 s for 25 cycles; and final extension at 72 °C for 7 min, while the profile of the nested PCR differed in an annealing temperature at 51 °C and a total of 35 cycles. Amplification products (5 µL) were mixed with GelRed™ stain and then visualized on a 1.5% agarose gel after 20 min at 90 V.

All amplification products were purified using the PCR Product Purification Kit (Roche, Mannheim, Germany). After sequencing (Microsynth AG, Balgach, Switzerland), the resulting sequence data were checked and edited using Geneious v. 2021.1.1 (<https://www.geneious.com>). The final sequences were then distinguished by identifying their closest matches in GenBank [31] using the NCBI nucleotide BLAST search. Avian haemosporidian parasites were additionally identified using the BLAST search of the MalAvi database [18]. If sequences differed in at least one basepair, it was considered a new lineage and naming was performed according to the system used for the MalAvi database: the first three letters of the bird genus, the first three letters of the species name, followed by a number (e.g., XENPOL01 from *Xenopirostris polleni*). In the text, haemosporidian lineage names were always accompanied by an abbreviation of its parasite genus (p: *Plasmodium*, h: *Haemoproteus*, l: *Leucocytozoon*). Newly amplified sequences of trypanosomes and filarioid nematodes were named according to their closest match in GenBank and given an isolate abbreviation for easier identification. All newly detected parasite lineages/sequences were deposited in GenBank (accession numbers OP006583–OP006613; Table S2 in Supplementary Materials).

Phylogenetic analyses of haemosporidian lineages, trypanosomes and filarioid nematodes detected in this study were performed using MEGA v.10.2 [32]. The dataset used for the first phylogenetic reconstruction consisted of cytochrome b sequences of haemosporidian lineages obtained in this study and reference sequences of different morphospecies of

Haemosporida downloaded from GenBank, each trimmed to 462 bp to ensure consistency in sequence length. A cytochrome b sequence from *Theileria annulata* (KF732030) was used as an outgroup. The second tree included all 18S rRNA sequences of *Trypanosoma* lineages detected in the sample set and sequences of homologous *Trypanosoma* sequences from GenBank, each trimmed to 745 bp. The human pathogen *Trypanosoma brucei* (AF306777) was used as an outgroup. The third dataset consisted of all sequences of microfilariiae detected in this study. Partial sequences of *cox1* and 28S rRNA were concatenated to perform phylogenetic analyses with a fragment of 1175–1342 bp length. *Ascaridia galli* (KY990014 and KT613888) was used as an outgroup. Phylogenies were generated by implementing the best-fitting model, which was identified by MEGA v.10.2 for every parasite taxon. All maximum likelihood methods were performed using 1000 replicates. The resulting phylograms were viewed and edited with MEGA v.10.2 [32].

3. Results

The molecular screening (multiplex and nested PCR) of 131 blood samples of different Corvoidea species allowed the detection of haemosporidian infections in 71 samples (54.2%) and *Trypanosoma* infection in 28 samples (21.4%). DNA of filarioid nematodes was detected in seven samples (5.3%) (Table 1). In 14 of the 56 blood smears obtained, gametocytes of Haemosporida were detected. Just one blood smear contained microfilariiae (1.8%), whereas two blood smears revealed trypanosomes (3.6%). The vangas showed the highest prevalence for haemosporidian parasites with 79.7%, whereas most of the *Trypanosoma* infections (27.5%) were detected in samples of *Terpsiphone mutata* (Monarchidae). *Leucocytozoon* was the only parasite taxon detected in blood samples of the ashy cuckoo shrike (Campephagidae), whereas samples of the *Dicrurus forficatus* (Dicruridae) showed only one infection with *Trypanosoma*. All kinds of parasite taxa (Haemosporida, *Trypanosoma* and microfilariiae) were detected in the *Terpsiphone mutata*, *Newtonia amphicroa* and *Tylas eduardi* samples, although *Haemoproteus* infections were absent in *Terpsiphone mutata* samples.

Table 1. Dataset of Corvoidea species examined in this study. Absolute numbers of blood smears (Nbs) and blood samples (N) are given. Results of parasite detections are listed for haemosporidian parasites, trypanosomes and filarioid nematodes, respectively. For each parasite taxon, percentages (%) of infected samples identified with microscopic method (Pmic) and molecular methods (Pmol), along with the name of the identified lineage/sequence, are given. “-” indicates that percentages were not measurable due to a lack of samples or that no sequence was amplified. If the parasite lineage/sequence was detected more than once, sample sizes are given in brackets. Newly detected lineages/sequences are given in bold letters.

bird species	Nbs	N	Haemosporida			<i>Trypanosoma</i>			microfilariiae		
			Pmic	Pmol	lineages (n)	Pmic	Pmol	sequence (n)	Pmic	Pmol	sequence (n)
Campephagidae											
<i>Coracina cinerea</i>	3	4	25	75	IFOUOMI07 (3)	0	0	-	0	0	-
Dicruridae											
<i>Dicrurus forficatus</i>	2	8	0	0	-	0	12.5	<i>T. avium</i> CORVOID2	0	0	-
Monarchidae											
<i>Terpsiphone mutata</i>	21	40	4.8	12.5	pCOPALB03 (2), ICINSOV02, ITERMUT01 , one unidentified <i>Leucocytozoon</i> double infection	0	27.5	<i>T. anguiformis</i> CORVOID03 (2), <i>T. avium</i> CORVOID1 , <i>T. avium</i> CORVOID3 , <i>T. avium</i> (KT728402) (5), <i>T. avium</i> CORVOID4 (2)	0	2.5	<i>Aproctella alessandroi</i> (1)

Table 1. Cont.

bird species	Nbs	N	Haemosporida		Trypanosoma			microfilariae			
			Pmic	Pmol	lineages (n)	Pmic	Pmol	sequence (n)	Pmic	Pmol	sequence (n)
Vangidae											
<i>Calicalicus madagascariensis</i>	5	12	0	58.3	pBERZOS01, IANLAT11, ICALMAD01 (2), ICALMAD02, ICALMAD03, ICALMAD04, IHYPMA02	0	25	<i>T. avium</i> (KT728402) (2), <i>Trypanosoma</i> sp. CORVOID01	0	0	-
<i>Cyanolanius madagascarinus</i>	0	4	-	50	pBERZOS01, pFOUMAD03, hCYAMAD01, ICYAMAD02	-	25	double infection	-	0	-
<i>Hypositta corallirostris</i>	0	1	-	100	<i>Leucocytozoon</i> sp. double infection	-	0	<i>T. avium</i> CORVOID4	-	0	-
<i>Leptopterus chabert</i>	2	2	100	100	hLEPCHA01 (2), ILEPCHA02, ILEPCHA03	50	0	0	0	0	-
<i>Mystacornis crossleyi</i>	0	6	-	16.7	<i>Haemoproteus micronuclearis</i> RBQ11	-	0	0	-	50	<i>Onchocercidae</i> sp. CROSSBAB (1), <i>Onchocercidae</i> sp. CROSSBAB2 (2)
<i>Newtonia amphichroa</i>	12	26	8.3	76.9	pNEWAM05 (2), pNEWAM01 (4), pNEWAM07 (3), hNEWAM04 (6), IFOMAD01, INEWAM08	0	7.7	<i>Trypanosoma</i> sp. CORVOID02, <i>T. avium</i> (KT728401)	8.3	7.7	<i>Onchocercidae</i> sp. DANEW (2)
<i>Newtonia brunneicauda</i>	5	12	100	100	pNEWAM06, hNEWBR01, hNEWBR02, hNEWBR03, hNEWBR04, hNEWBR05 (4), hNEWBR07, INEWAM03 (5), INEWBR06, IZOMAD01	0	33.3	<i>T. avium</i> CORVOID2, <i>T. avium</i> CORVOID1 (2), <i>T. avium</i> (KT728402)	0	0	-
<i>Pseudobias wardi</i>	1	4	100	100	<i>Plasmodium relictum</i> GRW04, hPSEWAR01 (2), hPSEWAR03, IPSEWAR02	0	50	<i>T. avium</i> (KT728401), <i>T. anguiformis</i> CORVOID03	0	0	-
<i>Tylas eduardi</i>	2	9	50	77.8	<i>Plasmodium relictum</i> GRW04 (3), hTYLEDU01, hTYLEDU04, ICALMAD01 (2), ITYLEDU02 (2), ITYLEDU03	0	22.2	<i>T. lewisi</i> (GU252209), <i>T. avium</i> (KT728402)	0	11.1	<i>Splendidofilaria bartletti</i> TYLAS (1)
<i>Vanga curvirostris</i>	2	2	100	100	pNEWAM07, hVANCUR01, IVANCUR02 (2)	50	50	<i>T. avium</i> CORVOID4	0	0	-
<i>Xenopirostris polleni</i>	1	1	0	100	pNEWAM07, IXENPOL01	0	0	0	0	0	-
total Vangidae	30	79	43.3	79.7		6.6	20.3		33.3	7.6	
total for the four families	56	131	26.8	54.2		3.6	21.4		1.8	5.3	

3.1. Microscopic, Molecular, and Phylogenetic Analyses of Haemosporidian Parasites

A total of 44 haemosporidian lineages (seven *Plasmodium*, 15 *Haemoproteus* and 22 *Leucocytozoon* spp.) were detected in the blood samples of birds. The majority of *Coracina cinerea* samples (n = 3) contained exclusively DNA of *Leucocytozoon* lineage IFOUOMI07. Gametocytes of this parasite were microscopically detected in blood smears (Figure 1k).

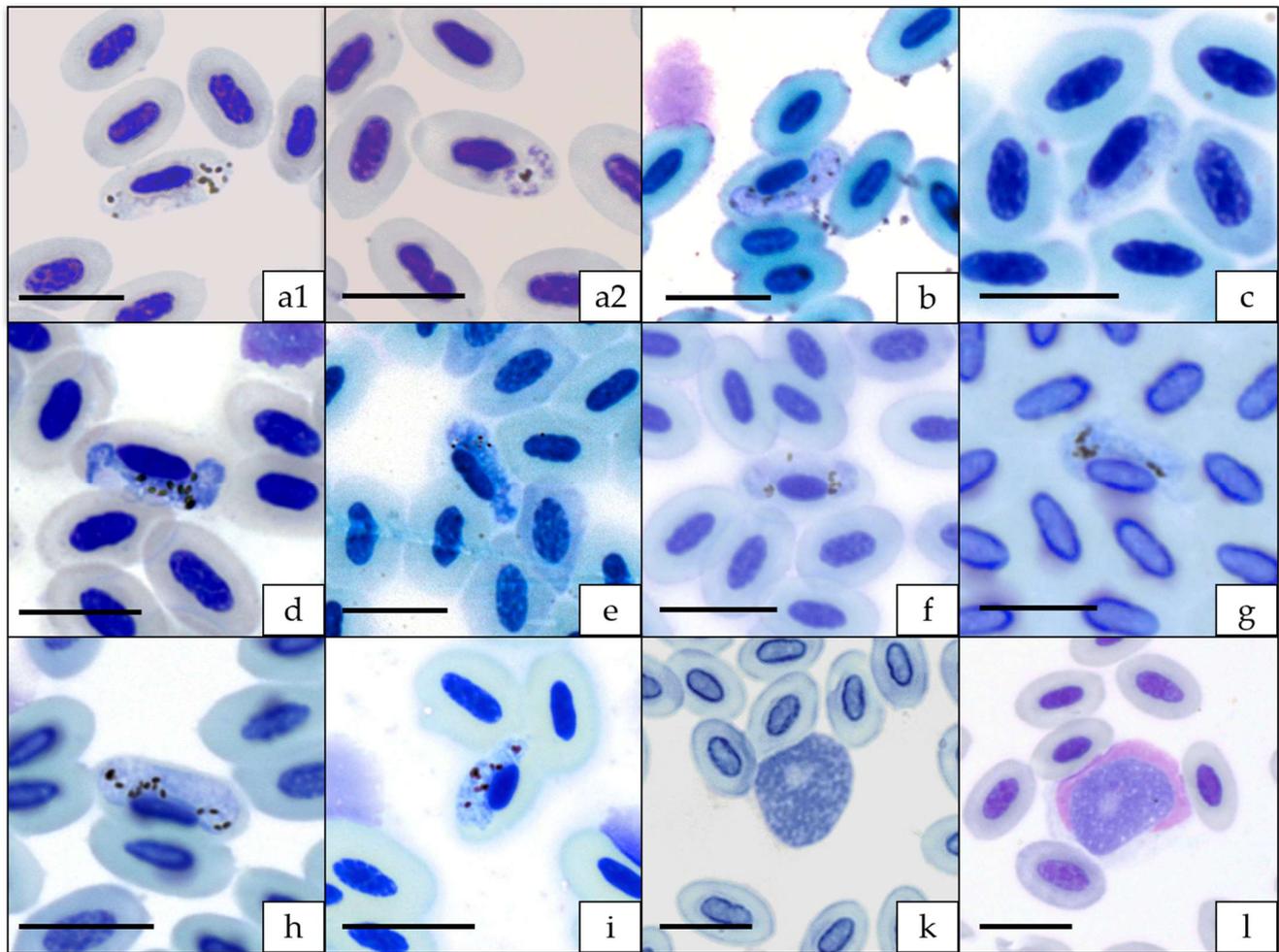


Figure 1. Blood stages of haemosporidian parasites of Corvoidea species from Madagascar. (a1) macrogametocyte and (a2) erythrocytic meront of *Plasmodium* pCOPALB03 from *Terpsiphone mutata*; (b): *Haemoproteus* hLEPCHA01 from *Leptopterus chabert*; (c): *Haemoproteus* hNEWAM04 from *Newtonia amphicroa*; (d): *Haemoproteus* hNEWBR03 from *Newtonia brunneicauda*; (e): *Haemoproteus* hNEWBR04 from *Newtonia brunneicauda*; (f): *Haemoproteus* hNEWBR05 from *Newtonia brunneicauda*; (g): *Haemoproteus* hNEWBR07 from *Newtonia brunneicauda*; (h): *Haemoproteus* hPSEWAR01 from *Pseudobias wardi*; (i): *Haemoproteus* hVANCUR01 from *Vanga curvirostris*; (k): *Leucocytozoon* IFOUOMI07 from *Coracina cinerea*; (l): *Leucocytozoon* IVANCUR02 from *Vanga curvirostris*. Blood smears were fixed with absolute methanol and stained with Giemsa. Scale bar = 10 μ m.

All samples of *Dicrurus forficatus* were negative for haemosporidian infection (Table 1). Five samples (12.5%) of *Terpsiphone mutata* contained DNA of *Plasmodium* sp. (pCOPALB03; n = 2) or *Leucocytozoon* spp. (ICINSOV02, ITERMUT01 and one unidentified mixed infection). Gametocytes and meronts were microscopically detected in one blood smear referring to a blood sample containing DNA of pCOPALB03 (Figure 1(a1,a2)). *Leucocytozoon* infections were missed using only multiplex PCR (See Table S1 in Supplementary Materials). Vangas showed the highest diversity of haemosporidian parasites and high prevalences for all three genera, with 49.4% *Plasmodium*, 45.6% *Haemoproteus* and 51.9% *Leucocytozoon*, respectively (Figure 2). Most of the infected samples contained mixed infections (60.4%), with 41.3% double infections, 14.3% triple infections and 4.8% quadruple infections. All combinations of mixed infections (P/H; P/L; H/L) occurred with similar frequencies. Gametocytes of eight *Haemoproteus* and two *Leucocytozoon* lineages were microscopically detected (Figure 1b–l).

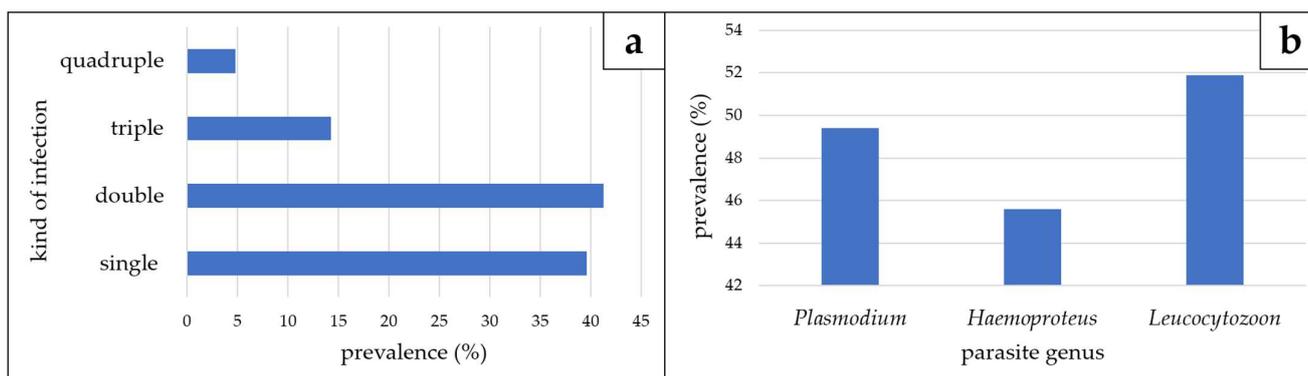


Figure 2. Kind of infection (a) and parasite prevalence (b) detected in species of the Vangidae examined throughout this study (n = 79).

The majority of haemosporidian lineages (n = 40), of which 15 were detected for the first time in this study, were isolated from vangas (Table 2). A high amount of the *Newtonia amphicroa* samples (34.6%) were found to be infected with three different *Plasmodium* lineages (pNEWAM05, pNEWAM01 and pNEWAM07), the highest diversity of *Plasmodium* lineages detected in our examined bird species. For *Haemoproteus* lineages, *Newtonia brunneicauda* showed the highest diversity, with six different lineages (hNEWBR01-05 and hNEWBR07) and a high prevalence for *Haemoproteus* (75%). *Calicalicus madagascariensis* was infected with six lineages of *Leucocytozoon* (IANLAT11, ICALMAD01-04 and IHYPMA02), thereby showing the highest diversity, with a prevalence of 58.3%. Just a few of the haemosporidian lineages were shared by different vanga species (Table 2). It is noteworthy that the vast majority of lineages detected throughout this study have been found exclusively on Madagascar. *Plasmodium relictum* (pGRW04) is the only lineage detected worldwide, whereas *Haemoproteus micronuclearis* hRBQ11 and *Leucocytozoon* lineage IANLAT11 have also been detected in African birds. IFOMAD01 has been detected in birds on Madagascar, as well as in various *Foudia* species (Ploceidae) on Mayotte and Comoros, and in *Hypsipetes madagascariensis* (Pycnonotidae) from Madagascar.

Table 2. Haemosporidian lineages found in our study. Isolation source is given as bird species name and total number of detections within the species (N). Furthermore, the percentage (%) of the lineage in the absolute infected samples of the bird species with regard to the parasite genus is also listed. Data of other bird hosts and sites of those hosts were drawn from the MalAvi database (as of July 2022). “-” indicates that no other host has been reported. Bold text indicates lineages for which gametocytes were detected in blood smears.

genus	lineage name	bird species	N	percentage (%)	other hosts	site
<i>Plasmodium</i>	BERZOS01	<i>Calicalicus madagascariensis</i>	1	33.3	<i>Xanthomixis zosterops</i> , <i>Bernieria madagascariensis</i>	Madagascar
	COPALB03	<i>Terpsiphone mutata</i>	2	100	<i>Copsychus albospectularis</i>	Madagascar
	FOUMAD03	<i>Cyanolanius madagascarinus</i>	1	50	11 species	Madagascar
	P. relictum (GRW04)	<i>Tylas eduardi</i>	3	50	91 species	worldwide
		<i>Pseudobias wardi</i>	1	50		
	NEWAM05	<i>Newtonia amphicroa</i>	2	11.1	<i>Bernieria madagascariensis</i> , <i>Foudia omissa</i>	Madagascar
	NEWAM01	<i>Newtonia amphicroa</i>	4	22.2	-	
		<i>Newtonia brunneicauda</i>	1	16.6		
	NEWAM07	<i>Newtonia amphicroa</i>	3	16.6	-	
		<i>Vanga curvirostris</i>	1	100		
	<i>Xenopirostris polleni</i>	1	100			

Table 2. Cont.

genus	lineage name	bird species	N	percentage (%)	other hosts	site
<i>Haemoproteus</i>	CYAMAD01	<i>Cyanolanius madagascarinus</i>	1	50	-	
	LEPCHA01	<i>Leptopterus chabert</i>	2	100	-	
	NEWAM04	<i>Newtonia amphicroa</i>	6	66.6	-	
	NEWBR01-03,04	<i>Newtonia brunneicauda</i>	1	8.3	-	
	NEWBR05	<i>Newtonia brunneicauda</i>	4	33.3	-	
	NEWBR07	<i>Newtonia brunneicauda</i>	1	8.3	-	
	PSEWAR01	<i>Pseudobias wardi</i>	2	40.0	-	
	PSEWAR03	<i>Pseudobias wardi</i>	1	20	-	
	<i>H. micronuclearis</i> (RBQ11)	<i>Mystacornis crossleyi</i>	1	100	<i>Quelea quelea</i> , <i>Q. erythroptus</i> , <i>Ploceus melanocephalus</i> , <i>Foudia omissa</i> , <i>F. madagascariensis</i> (Ploceidae)	sub-Saharan Africa
	TYLEDU01	<i>Tylas eduardi</i>	2	66.6	-	
TYLEDU04	<i>Tylas eduardi</i>	1	33.3	-		
VANCUR01	<i>Vanga curvirostris</i>	1	100	-		
<i>Leucocytozoon</i>	ANLAT11	<i>Calicalicus madagascariensis</i>	1	9	<i>Andropadus latirostris</i>	Ghana
	CALMAD01	<i>Tylas eduardi</i>	2	22.2	-	
		<i>Calicalicus madagascariensis</i>	2	18.1	-	
	CALMAD02-04	<i>Calicalicus madagascariensis</i>	1	9	-	
	CINSOV02	<i>Terpsiphone mutata</i>	1	25	<i>Cinnyris sovimanga</i>	Madagascar
	CYAMAD02	<i>Cyanolanius madagascarinus</i>	1	100	-	
	FOMAD01	<i>Newtonia amphicroa</i>	1	33.3	<i>Foudia madagascariensis</i> , <i>F. omissa</i> , <i>F. eminentissima</i> , <i>Hyppipetes madagascariensis</i>	Comoros, Mayotte
	FOUOMI07	<i>Coracina cinerea</i>	3	100	-	
	HYPMA02	<i>Calicalicus madagascariensis</i>	1	9	<i>Foudia omissa</i> , <i>F. madagascariensis</i> , <i>Hyppipetes madagascariensis</i> , <i>Neodrepanis coruscans</i> , <i>Philepitta castanea</i>	Madagascar
	LEPCHA02-03	<i>Leptopterus chabert</i>	1	25	-	
	NEWAM03	<i>Newtonia brunneicauda</i>	5	71.4	<i>Foudia omissa</i> , <i>N. amphicroa</i>	Madagascar
	NEWAM08	<i>Newtonia amphicroa</i>	1	33.3	-	
	NEWBR06	<i>Newtonia brunneicauda</i>	1	14.3	-	
	PSEWAR02	<i>Pseudobias wardi</i>	1	33.3	-	
	TERMUT01	<i>Terpsiphone mutata</i>	1	25	-	
	TYLEDU02	<i>Tylas eduardi</i>	2	22.2	-	
	TYLEDU03	<i>Tylas eduardi</i>	1	11.1	-	
VANCUR02	<i>Vanga curvirostris</i>	2	100	-		
XENPOL01	<i>Xenopirostris polleni</i>	1	100	-		
ZOMAD01	<i>Newtonia brunneicauda</i>	1	14.3	<i>Zosterops maderaspatanus</i>	Madagascar	

All haemosporidian genera appeared as monophyletic groups (Figure 3). Within the *Plasmodium* subtree pNEWAM05 and pCOPALB03 formed an independent clade. They differed in 18 bp on the 662 bp fragment of cytochrome b which was used for phylogenetic analysis. pNEWAM07 and pBERZOS01 formed another clade. These lineages differed in only two bp but were isolated from completely different bird species. pFOUMAD03 differed in only one single basepair from *Plasmodium relictum* pGRW04, whereas pNEWAM01 showed no close relationship to the other lineages detected in the Corvoidea samples.

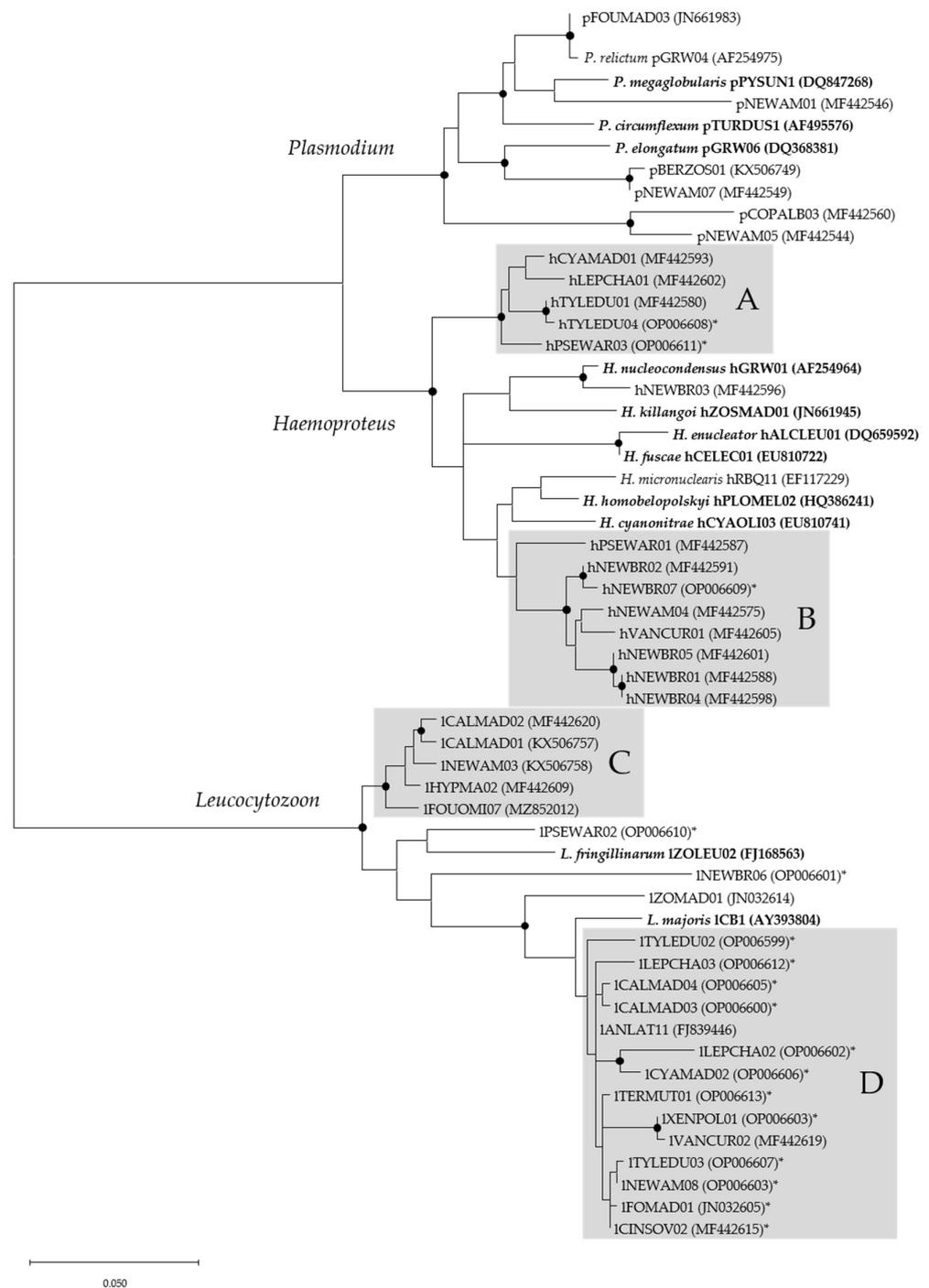


Figure 3. Phylogenetic relationship of 56 mitochondrial *cytb* lineages of haemosporidian parasites detected in blood samples of birds belonging to the superfamily Corvoidea on Madagascar, along with sequences of different morphospecies, constructed using maximum likelihood (GTR + G). Dots on nodes indicate bootstrap values >70%. In brackets are given GenBank accession numbers for all lineages. Morphospecies are written where linked to the characteristic lineages. Those in bold were not detected throughout this study. New lineages that were found for the first time in the present study are marked with an asterisk (*). Grey boxes (A–D) indicate groups of closely related *cytb* lineages.

3.2. Microscopic, Molecular and Phylogenetic Analyses of Trypanosomes

Within the *Haemoproteus* subtree, the lineages detected throughout this study mainly appeared in two distinct clades (Figure 3A,B). The lineage hNEWBR03 showed the greatest

homology with *H. nucleocondensus* hGRW01 with eight basepair differences. *Haemoproteus micronuclearis* hRBQ11, which was detected once in the sample set, was not closely related to the other lineages of *Haemoprotues* isolated from Vangidae in this study.

The majority of *Leucocytozoon* lineages detected in this study also formed two separate clades (Figure 3C,D). Clade D showed a close relationship to the morphospecies *Leucocytozoon majoris* ICB1. The lineages LPSEWAR02, INEWBR06 and IZOMAD01 showed no close relationship to other lineages included in this analysis.

The highest prevalence for *Trypanosoma* infections was found in the family Monarchidae (*Terpsiphone mutata*) (27.5%), followed by vangas (20.3%), with a single confirmation of *Trypanosoma* DNA in *Dicrurus forficatus* (12.5%). Two microscopic detections of trypanosomes were made in two samples of Vangidae: one in *Leptopterus chabert* and one in *Vanga curvirostris*. However, the trypanosome in the *L. chabert* could not be determined more precisely by molecular methods because amplification of the gene fragment failed. The parasite in *V. curvirostris* was identified as the newly described *T. avium* isolate CORVOID4 (OP006598; Figure 4).

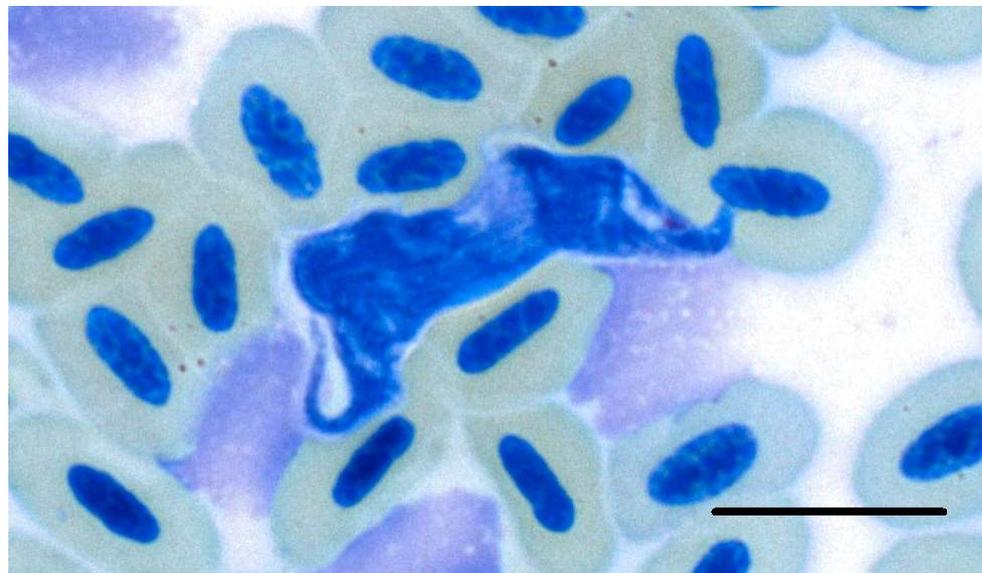


Figure 4. Trypomastigote form of *Trypanosoma avium* isolate CORVOID4 (OP006598) detected in a blood smear of *Vanga curvirostris* in Madagascar. Scale bar = 10 μ m.

Overall, 10 different *Trypanosoma* sequences of the SSU rRNA were detected in the Malagasy Corvoidea samples (Table 1). The sequences were assigned to three known species (*T. avium*, *T. anguiformis* and *T. lewisi*); two sequences still need to be identified to species level (*T. sp.* CORVOID01 and 02). Three sequences were 100% homologous to previously published sequences (*T. avium* (KT728401 and KT728402) and *T. lewisi* (GU252209)). Five of the detected *Trypanosoma* sequences were shared between different bird genera. *Trypanosoma avium* (KT728402) was the most abundant detected sequence, isolated from five *Terpsiphone mutata* and four vangas. The highest sequence richness in a single bird species was found in *T. mutata*, whereas the highest prevalence (50%) was found in *Pseudobias wardi* (n = 2) and *V. curvirostris* (n = 1). Exceptionally low prevalence (7.7%) was detected in *Newtonia amphicroa* (n = 26). The only sequence detected in *Dicrurus forficatus* was the *T. avium* isolate CORVOID2, a sequence additionally found once in *Newtonia brunneicauda*.

Phylogenetically, all sequences formed a monophyletic branch (Figure 5), except for the sequence homologous to *T. lewisi* (GU252209), which formed another branch with the complete homologous sequences of *T. blanchardi* (AY491764), *T. musculi* (AJ223568) and *T. niviventerae* (AB242274). These sequences have previously been detected exclusively in rodents and are described as *T. lewisi-like* trypanosomes [33]. Sequences forming the other branch have to date been exclusively detected in bird hosts. This branch was again

divided into two branches. One contained sequences assigned to the species *T. avium*, the other contained sequences related to *T. anguiformis* or an unknown species. Although the *Trypanosoma* sp. isolate CORVOID01 forms a clade with sequences of *T. anguiformis*, it is considered as an unknown species as this sequence differs in at least eight basepairs.

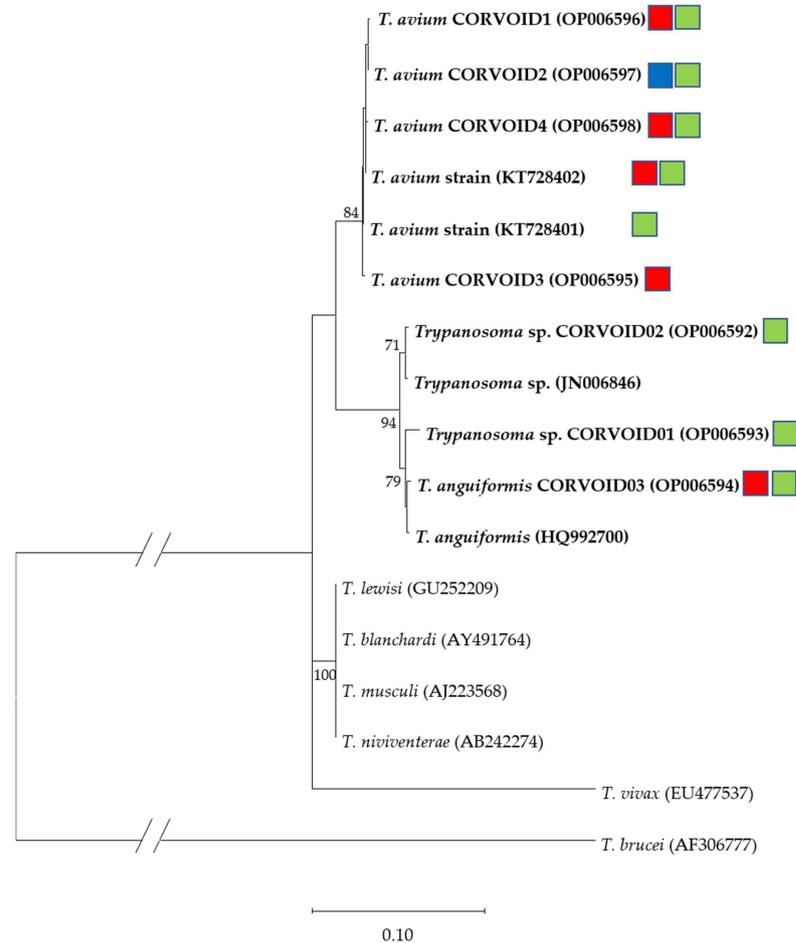


Figure 5. Phylogenetic relationship of *Trypanosoma* SSU rRNA sequences (Acc. No.) detected in Corvoidea species on Madagascar, along with highly homologous previously published sequences (Acc. No.) constructed using maximum likelihood (K2 + G). Bootstrap values >70% are given. Bold text indicates sequences exclusively isolated from birds. Bird families from which the lineages were isolated are indicated with color-coded squares. Red = Monarchidae; blue = Dicruridae; green = Vangidae.

3.3. Microscopic, Molecular, and Phylogenetic Analyses of Microfilariae

The amount of filarioid nematode infections was extremely low (5.3%). The highest prevalence was found in *Mystacornis crossleyi* (Vangidae), with three positive samples (50%; Table 1). Morphological detection was possible for the *Onchocercidae* sp. isolate DANEW in a blood smear of *Newtonia amphicroa* (Figure 6). One blood sample of the latter contained a mixed infection, identified by numerous double peaks in the chromatogram of the 28S rRNA sequence. The *cox1* sequence of this sample was identified as *Onchocercidae* sp. isolate DANEW.

Four different 28S rRNA sequences were identified linked to five *cox1* sequences. The 28S rRNA of *Onchocercidae* sp. isolate CROSSBAB was found in three *M. crossleyi* samples, but two different *cox1* sequences were isolated from them (CROSSBAB and CROSSBAB2) with two basepair changes that distinguished these sequences. No sequence was shared between different bird species or families. No sequence was 100% homologous to previously published sequences.

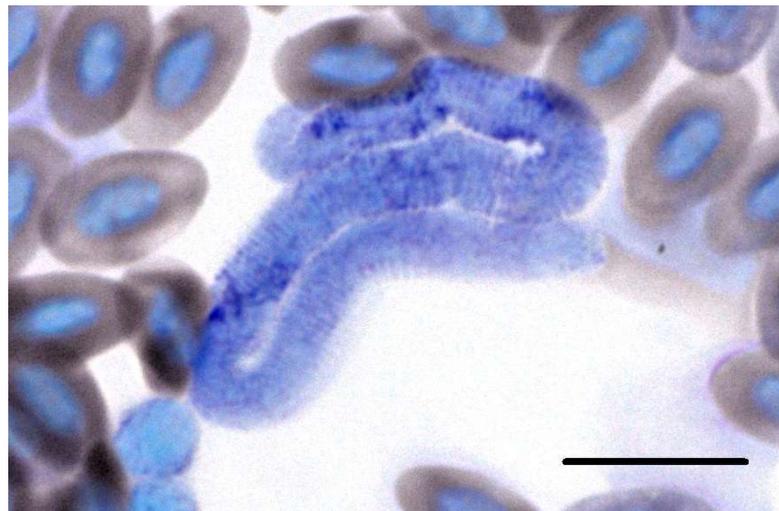


Figure 6. Larval stage (microfilaria) of *Onchocercidae* sp. isolate DANEW (OP006587 and OP006583) isolated from *Newtonia amphicroa* (Vangidae) in Madagascar. Scale bar = 10 μ m.

Phylogenetic analysis (Figure 7) revealed close relatedness of the isolate from *Tylas eduardi* to *Splendidofilaria bartletti*, a nematode detected in a single *Sylvia atricapilla* from Lithuania [22]. The *Onchocercidae* sp. isolates CROSSBAB and CROSSBAB2 were quite homologous, with just two basepair changes and formed an individual branch within the tree. All microfilariae isolated from Vangidae appeared in the same clade, whereas the isolate from *Terpsiphone mutata* (Monarchidae), *Aproctella alesandroi* isolate PARFLY was separated from them.

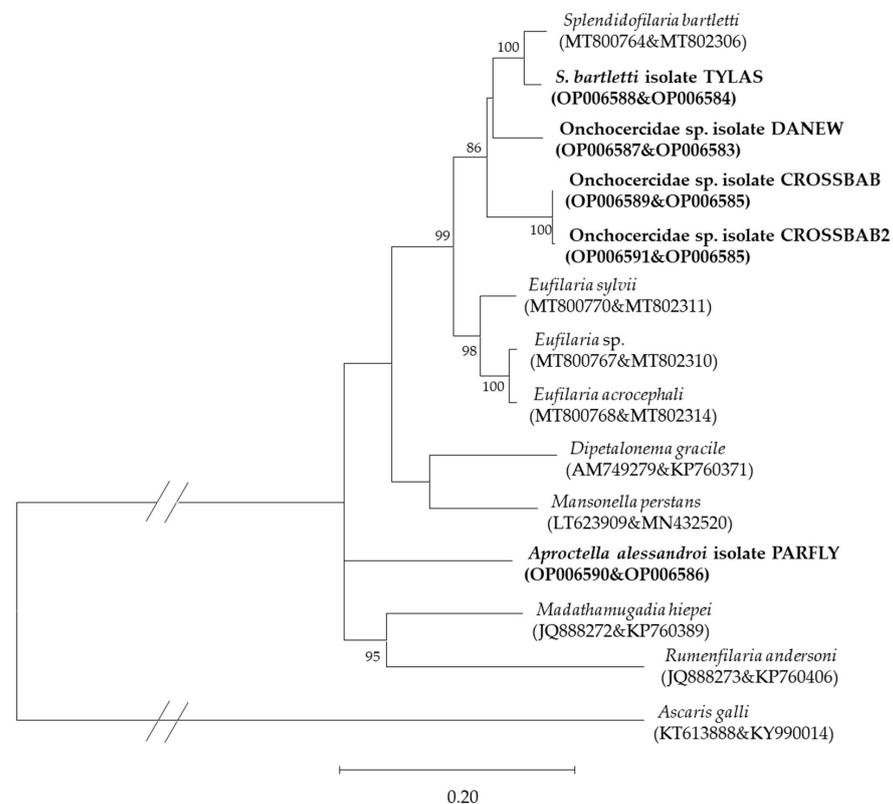


Figure 7. Phylogenetic relationship of concatenated filarioid nematode cox1 and 28S rRNA sequences (Acc. No.) detected in Corvoidea species on Madagascar, along with previously published sequences of avian filarioid nematodes, constructed using maximum likelihood (GTR + G + I). Bold text indicates sequences isolated in this study. Bootstrap values >70% are given.

4. Discussion

The parasite abundance and diversity detected in the Corvoidea species on Madagascar demonstrate how blood parasite infections can vary among closely related bird families or species.

4.1. Haemosporida

The prevalence of haemosporidian parasites varied greatly between the closely related Corvoidea species. Whereas vangas harbored an enormous diversity and high abundance of Haemosporida, the other examined families showed little prevalence of haemosporidian infections. The only exception was *Coracina cinerea* (Campephagidae), which had no infection with *Plasmodium* and *Haemoproteus* spp. but a high prevalence of *Leucocytozoon* spp. Three of the four blood samples examined contained DNA of *Leucocytozoon* lineage IFOUOMI07. This lineage has previously been found in one blood sample of *Foudia omissa* (Ploceidae) on Madagascar [34]. In our study, gametocytes were detected in blood smears of *C. cinerea*, which confirms that this bird species is a suitable host for IFOUOMI07. Since DNA of IFOUOMI07 was detected in only one of the 207 *Foudia* blood samples, and no gametocytes were found by Musa et al. [34], it is assumed that this single case might represent an abortive infection and that IFOUOMI07 is specialized on *Coracina cinerea*. Barraclough et al. [14] detected *Leucocytozoon coracinae* in *Coracina cinerea* blood smears. They described the parasite as a ‘typically small round morph’ that presumably occurs in the different species of Campephagidae [14]. We were not able to compare the gametocytes of IFOUOMI07 with those of *L. coracinae* as our detected gametocyte was not connected with the nucleus of the host cell, which is a prerequisite for the identification of the host species. However, it is possible that IFOUOMI07 does represent the *cytb* sequence of *L. coracinae*.

For *Dicrurus forficatus*, there were conflicting results with respect to Haemosporida prevalence. In our study, no haemosporidian lineage was detected (n = 8). However, Savage et al. [11,35] found gametocytes of *Haemoproteus dicruri* and *H. khani* in blood smears of 11 birds (n = 18). This difference might have been due to the absence of suitable vectors in the Maromizaha rainforest, or the low number of samples examined in this study. More data is needed to test these hypotheses.

Low haemosporidian prevalence in *Terpsiphone mutata* (Monarchidae) was not only found in our study (12.5%) but has also been reported in several other studies [11,12,15]. Greiner et al. [13] detected *Plasmodium* spp. in a single blood smear of *T. mutata*. However, no further information about absolute sample size or the morphology of the gametocytes or erythrocytic meronts are given. Twenty blood smears of Monarchidae species, presumably *T. mutata*, were examined by Raharimanga et al. [12]. In three blood smears (15%) gametocytes of *Plasmodium* or *Haemoproteus* species were detected but no further determination was provided. Savage et al. [11] examined a total of 90 blood smears of *T. mutata* for the presence of haematozoan parasites. They detected *Haemoproteus* species in three of the examined samples (3.33%). Again, further information about parasite species identity or morphology was missing. Ivanova et al. [15] examined nine *T. mutata* using both morphological and molecular methods. Neither method provided evidence of haemosporidian infection. In our study, we detected the *Plasmodium* lineage pCOPALB03 in two samples (5%) and two different *Leucocytozoon* lineages, as well as an unidentified mixed infection in three other samples. In contrast to Savage et al. [11], we did not detect any *Haemoproteus*. However, we were able to identify gametocytes, as well as erythrocytic meronts of pCOPALB03, and, therefore, demonstrated that *T. mutata* is a suitable host of this lineage. The species description of this parasite is still pending. The lineage itself has previously been detected in only a single blood sample of *Copsychus albospectularis* (Muscicapidae) on Madagascar [16]. Further studies are needed to rule out whether this parasite might be specialized on *T. mutata* and if it is restricted to Madagascar.

Considering a total of 11 species, we examined half of the existing Vangidae species. In contrast to the Corvoidea families, vangas showed high prevalence for haemosporidian

parasites (79.7%). This is in accordance with other studies that examined blood samples or blood smears of vangas [11,12,15,16]. Vangas harbored an enormous diversity of avian haemosporidian parasites, with six *Plasmodium*, 15 *Haemoproteus* and 18 distinct *Leucocytozoon* lineages detected in our sample set. The parasites found in this family seem, in most cases, to be highly specialized. *Haemoproteus* species are known, in general, to be highly specialized, whereas *Plasmodium* species tend more often to be generalists [16]. For *Leucocytozoon* species, very little information exists about specialization but there seem to be both highly specialized species and generalists [16,36,37]. Detailed examples of highly specialized lineages from our study are provided below.

Cyanolanius madagascarinus, for example, was found to be infected with hCYAMAD01 and ICYAMAD02. Both lineages were found exclusively in this bird species (Table 2). We assume that both lineages are specialized on this bird species and restricted to Madagascar. Savage et al. [11] examined a single blood smear of *C. madagascarinus* and detected gametocytes of *Haemoproteus vangii* and *Leucocytozoon lairdi*. These are probably morphospecies of the lineages we detected, but, as we did not have blood smears for morphological examination, we were not able to compare gametocytes.

Mystacornis crossleyi is a vanga species which showed low haemosporidian prevalence. We detected *Haemoproteus micronuclearis* (hRBQ11) in one blood sample (16.6%). This species is reported to be specialized on birds of the family Ploceidae [16]. Since such an infection in *M. crossleyi* seems to be very unlikely, it could be an abortive infection, which would mean that *M. crossleyi* is not a natural host. However, we had no blood samples to resolve this uncertainty. Savage et al. [11] examined two blood smears of *M. crossleyi* but did not detect gametocytes of Haemosporida. Ivanova et al. [15] detected the generalist *Leucocytozoon* lineage IFOMAD01 in a single sample of *M. crossleyi*. However, they were not able to detect gametocytes of this lineage in the blood smear of the bird. Thus, an abortive infection cannot be ruled out, or *M. crossleyi* was just an accidental host.

Newtonia amphicroa was the vanga with the highest number of blood samples in our study. Compared to the other Vangidae species, we detected a high diversity regarding the genus *Plasmodium*. pNEWAM05 seems to be a generalist restricted to Madagascar as this lineage was also isolated from bird species of other avian families [16]. However, pNEWAM07 might be specialized on Vangidae species, whereas pNEWAM01 might be specialized on *Newtonia* species. All lineages have been exclusively detected on Madagascar (Table 2). The *Haemoproteus* lineage NEWAM04 was the only one detected in *N. amphicroa* in this study. Gametocytes of this lineage were also found in blood smears (Figure 1c). Savage et al. [11] detected gametocytes of *H. vangii* in two of 18 blood smears of *N. amphicroa*. The comparison of the gametocytes of *H. vangii* and hNEWAM04 revealed many similarities, including fine pigment granules. However, the quality of our blood smears was not sufficiently good to compare all measurements and perform morphological determination to species level. We still predict that hNEWAM04 will represent the *cytb* sequence of *H. vangii*. It has been suggested that *H. vangii* has additional Vangidae species as hosts [11]. As hNEWAM04 seems to be specialized on *N. amphicroa*, other gametocytes found might be either cryptic species with similar morphology or *H. vangii* has different haplotypes which show specialization on different vanga species. Further studies are needed to resolve this question.

Just two samples of *N. amphicroa* (7.7%) contained DNA of *Leucocytozoon* species. One lineage, IFOMAD01, has recently been reported to be a highly abundant generalist on Madagascar [34]. However, this was the only finding of IFOMAD01 in our study. It is possible that, in this case, there is also an abortive infection and that the Corvoidea are basically resistant to this parasite. In contrast to our findings, Ivanova et al. [15] also detected the lineage hNEWAM02 in samples of *N. amphicroa*, as well as in *N. brunneicauda* samples and lNEWAM03 in two of three *N. amphicroa* samples. hNEWAM02 was not detected in any sample of our study, whereas lNEWAM03 was frequently detected in *N. brunneicauda* samples (41.7%) but not in *N. amphicroa* (n = 26). lNEWAM03 has additionally been detected only in a single *Foudia omissa* sample on Madagascar [16] to date.

Due to the limited data in that study, the lineage was grouped with other *Leucocytozoon* lineages, and it was predicted that these might represent generalist species. Based on the newly available data from the present study, the most likely hypothesis is that the *Leucocytozoon* lineages of Vangidae, including INEWAM03, are specialized and may even represent distinct species. With respect to this assumption, the finding of INEWAM03 in *Foudia omissa* might, therefore, represent an abortive infection and, since *N. amphicroa* is hard to distinguish from *N. brunneicauda*, the results obtained by Ivanova et al. [15] should also be revised to corroborate their validity.

Newtonia brunneicauda was found to be infected with another, probably specialized, *Leucocytozoon* lineage—INEWBR06. However, there was just a single detection of that lineage and, therefore, no reliable statement can be made. The diversity of *Plasmodium* and *Leucocytozoon* lineages in *N. brunneicauda* was rather low, especially in contrast to the *Haemoproteus* lineages found. Six lineages were detected in the 12 blood samples of *N. brunneicauda* in this study with hNEWBR05 being the most abundant lineage. Gametocytes of hNEWBR03-5 and hNEWBR07 were additionally detected in blood smears which confirms that *N. brunneicauda* is a competent host for those parasites. According to the phylogenetic analyses the six *Haemoproteus* lineages might be assigned to at least three different species, one consisting of hNEWBR03, one of the haplotypes hNEWBR02 and 07, and a third comprising the lineages hNEWBR01, 04 and 05 (Figure 3 and Table S3 in Supplementary Materials). However, comparison of macrogametocytes revealed morphological differences between hNEWBR04 and 05. hNEWBR01 and 04 may represent a separate species as well as hNEWBR05, which seems to be another specialized *Haemoproteus* species of *N. brunneicauda*. Further species determinations are needed to confirm this hypothesis.

Tylas eduardi samples harbored all three haemosporidian genera. *Plasmodium relictum* (pGRW04) was found in 33.3% of the samples. With hTYLED01/04 and ITYLEDU02/03, *T. eduardi* probably contained four specialized parasite lineages. The lineage ICALMAD01, found twice in *T. eduardi*, was additionally identified in *Calicalicus madagascariensis*. It was the only *Leucocytozoon* lineage shared between Vangidae species. As findings of this lineage have been restricted to Vangidae [15], possible specialization at bird family level is still predicted. Savage et al. [11] described *Leucocytozoon lairdi* from various Vangidae species, including *T. eduardi*. It can be assumed that ICALMAD01 might be the cytochrome b sequence of *L. lairdi*. Because no gametocytes of ICALMAD01 were found in this study, this hypothesis cannot be verified.

Calicalicus madagascariensis harbored the highest diversity of *Leucocytozoon* lineages of all the examined bird species. Besides ICALMAD01 there were three other lineages (ICALMAD02-04) that might be specialized on *C. madagascariensis*. Phylogenetic analyses revealed a remarkably close relationship of ICALMAD01 and 02, as well as ICALMAD03 and 04. Due to low genetic difference (2 bp), ICALMAD03 and ICALMAD04 might be haplotypes of one species. Another *Leucocytozoon* lineage detected in *C. madagascariensis* was IHYPMA02. This lineage might be a highly abundant generalist in Madagascar [36], like IFOMAD01. However, as this was the only finding of IHYPMA02 in our study, resistance of all Corvoidea species against this parasite cannot be ruled out.

Vanga curvirostris is a suitable host for hVANCUR01 and IVANCUR02 as we detected gametocytes of both lineages in blood smears of this bird species. hVANCUR01 might be the *cytb* sequence of *Haemoproteus madagascariensis*, a species described and found exclusively in *V. curvirostris* by Savage et al. [38], as the morphology of the gametocytes is similar. In contrast to the assumption of Savage et al. [38] that this parasite will be found in the different vanga species on Madagascar, we predict specialization of this parasite species on *V. curvirostris*. Due to morphological similarities, we also assume that the *Leucocytozoon* lineage IVANCUR02 might represent *L. benetti*. This species was described by Savage et al. [38] and was detected in *V. curvirostris* and the helmet vanga, *Euryceros prevostii* [38]. This parasite might also be specialized on vangas. However, as *Leucocytozoon* species often show extensive cryptic diversity [37], it cannot be ruled out that IVANCUR02 represents a separate species specialized on *V. curvirostris*.

The only *Plasmodium* lineage detected in *V. curvirostris* samples in this study was pNEWAM07. This lineage was detected in several vanga species and is, therefore, assumed to be specialized on this bird family. Savage et al. [39] detected a low prevalence of *P. parvulum* at (0.3%) in three vanga species, including *V. curvirostris*. pNEWAM07 might be the matching *cytb* sequence for *P. parvulum*. However, as we were not able to find gametocytes or erythrocytic meronts in our samples, this hypothesis still needs to be verified in further studies.

Other examples of potentially specialized haemosporidian lineages were found in *Leptopterus chabert* (hLEPCHA01, ILEPCHA02 and ILEPCHA03), *Pseudobias wardi* (IPSEWAR02 and hPSEWA01/03) and *Xenopirostris polleni* (IXENPOL01). Further data are needed until reliable conclusions can be drawn.

4.2. *Trypanosoma*

Based on the assumption that *Trypanosoma* species are host-specific, at least 96 trypanosome species were formerly identified in birds, but this theory was rejected, as trypanosomes can be transmitted to multiple host bird species [40]. However, due to the pleomorphic character of this taxon, an accurate morphological description of *Trypanosoma* species is extremely difficult and studies on this topic are problematic [40]. Sehgal et al. [40] provided the first molecular-based evidence that a single morphospecies of *T. avium* can infect several avian species, consistent with the idea of low host-specificity for *Trypanosoma*. *T. avium* is described as a single species, although it has been identified in Africa, north America, and Europe, and even though the multiple sub-species differed in terms of morphological criteria [41]. Morphological studies showed that *T. avium* parasitizes at least 13 passeriform bird species in sub-Saharan Africa [41] and many bird hosts belonging to the Nectariniidae family showed a high prevalence [40]. The predicted low host-specificity could be confirmed for the four haplotypes of *T. avium* identified in this study, of which the most abundant sequence, *T. avium* (KT728402), was isolated from five *Terpsiphone mutata* samples and four Vangidae samples. Savage et al. [11] also detected *T. avium* in three vanga species and *Atelornis pittoides* (Brachypteraciide) on Madagascar.

Currently, *Trypanosoma anguiformis* has only been reported in Ghana where it was found in two different areas, with an overall prevalence of six out of 104 infected bird species (5.8%) [20]. We found haplotypes of this species twice in *Pseudobias wardi* (Vangidae) and once in *Terpsiphone mutata* (Monarchidae), which are both endemic to Madagascar. Vectors for *Trypanosoma* species are louse flies (Hippoboscidae) and biting midges (Ceratopogonidae) [42,43]. *T. anguiformis* may have reached Madagascar from mainland Africa via infected vectors or bird hosts and haplotypes infecting the Malagasy avian fauna may have been developed.

The single infection of *Tylas eduardi* with *Trypanosoma lewisi* is surprising. *T. lewisi* belongs to a complex of obligatory rodent parasites found worldwide [44]. *Trypanosoma* can be cyclically transmitted via peroral contamination by flea feces or accidental ingestion of fleas [45]. As *T. eduardi* is primarily insectivorous [2], an infection could have occurred due to an ingestion of fleas. Even though *T. lewisi* trypanosomes are considered highly host specific, there have been studies of *T. lewisi* or *T. lewisi-like* infecting other species, such as monkeys [44], although these interspecies cross-infections rarely occur naturally [45]. We assume that this *Tylas eduardi* individual was probably weakened by its *Plasmodium* and *Leucocytozoon* co-infection and developed this unusual infection. However, mechanical transmission and abortive infection cannot be ruled out.

Sehgal et al. [40] described the possibility of hosting more than one *Trypanosoma* species in one bird species; however, this could not be confirmed in this study.

For the birds for which blood smears as well as blood samples were available, molecular methods were overall more sensitive than microscopic methods in the detection of trypanosomes. This might be explained by the fact that trypanosomes are usually quite rare in peripheral blood vessels [46].

4.3. Microfilariae

Seven bird blood samples contained DNA of filarioid nematodes (5.3%). These sequences could be assigned to five different specimens. Other studies have found DNA of *Cardiofilaria* and *Eufilaria* in Campephagidae, whilst DNA of *Chandlerella* was detected in Dicuridae but not in vangas [47]. In our study, we detected four DNA sequences in five samples of vanga species: *Onchocercidae* sp. isolate CROSSBAB/2, *Splendidofilaria bartletti* isolate TYLAS and *Onchocercidae* sp. isolate DANEW. A DNA sequence of the *Aproctella alessandroi* isolate PARFLY was found once in *Terpsiphone mutata* (Monarchidae). The results indicate that the nematodes are very host specific. Savage et al. [11] also found microfilariae in blood smears of Vangas, but more data about the species identity was not provided. In contrast to other studies [11,47], we did not detect any filarioid nematodes in samples of *Dicrurus forficatus*. However, the small dataset (n = 8) limited the possibility of detection.

For the other three species mentioned in the results, little information is available, not only on the possible transmitting vectors, but also on the first classification of the species and, therefore, their host type.

5. Conclusions

Corvoidea on Madagascar showed extreme differences in their parasite abundance, composition, and diversity. *Coracina cinerea* (Campephagidae), *Dicrurus forficatus* (Dicruridae) and *Terpsiphone mutata* (Monarchidae) showed a low susceptibility for avian haemosporidian parasites. However, the sample sizes for *Coracina cinerea* and *Dicrurus forficatus* were too small to enable final conclusions to be drawn. Except for single specialized parasites, such as pCOPLAB03 and IFOUOMI07, these members of the Corvoidea seem to be quite resistant to haemosporidian parasites. In contrast to potential resistance against Haemosorida, *Terpsiphone mutata* showed a high prevalence and diversity of *Trypanosoma* species. Vangas, in general, seemed to be more susceptible to all kinds of blood parasites as they harbored a variety of so-called host-specific haemosporidian parasites of all genera with high prevalence. Whereas trypanosomes seem to be generalists, the filarioid nematodes detected might be specialized. These findings offer interesting insights into the complex host-parasite interactions that have evolved in each family or species of the Corvoidea on Madagascar. Future studies on Madagascar with a larger dataset, including more species from the different families referred to in this study, are needed to test the possibility of co-existence between blood parasites or the specificity of the parasite-host and host-vector relationships.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14100888/s1>, Table S1: Haemosporidian detections in blood samples of Corvoidea species from Madagascar (n) using multiplex and nested PCR.; Table S2: Newly described lineages/sequences in our study. Accession number, target gene fragment and names are given.; Table S3: Genetic distances between *Haemoproteus* lineages isolated from *Newtonia brunneicauda* (Vangidae) on Madagascar based on a 462 bp fragment of cytochrome b.

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