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Avian malaria on Madagascar: prevalence, biodiversity and specialization of haemosporidian parasites ☆

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ABSTRACT

Previous studies about geographic patterns of species diversity of avian malaria parasites and others in the Order Haemosporida did not include the avian biodiversity hotspot Madagascar. Since there are few data available on avian malaria parasites on Madagascar, we conducted the first known large-scale molecular-based study to investigate their biodiversity. Samples (1067) from 55 bird species were examined by a PCR method amplifying nearly the whole haemosporidian cytochrome *b* gene (1063 bp). The parasite lineages found were further characterized phylogenetically and the degree of specialization was determined with a newly introduced host diversity index (Hd). Our results demonstrate that Madagascar indeed represents a biodiversity hotspot for avian malaria parasites as we detected 71 genetically distinct parasite lineages of the genera *Plasmodium* and *Haemoproteus*. Furthermore, by using a phylogenetic approach and including the sequence divergence we suspect that the detected haemosporidian lineages represent at least 29 groups i.e. proposed species. The here presented Hd values for each parasite regarding host species, genus and family strongly support previous works demonstrating the elastic host ranges of some avian parsites of the Order Haemosporida. Representatives of the avian parasite genera *Plasmodium* and *Leucocytozoon* tend to more often be generalists than those of the genus *Haemoproteus*. However, as demonstrated in various examples, there is a large overlap and single parasite lineages frequently deviate from this rule.

1. Introduction

Haemosporidian parasites are a diverse group of vector-transmitted blood-borne parasites, including the genera *Plasmodium, Haemoproteus* and *Leucocytozoon*, and are abundant in many bird families (Valkiunas, 2005; Bensch et al., 2009). Parasites of the Order Haemosporida occur worldwide, except in the Antarctic (e.g. Beadell et al., 2009; Bensch et al., 2009; Clark et al., 2014), but different geographic patterns of species diversity have already been identified by Clark et al.(2014) for avian haemosporidian parasites. They determined three biogeographic groups of haemosporidian diversity based on their avian richness: continental avian hotspot (300–1000 + spp.), continental avian non-hotspot (60–299 spp.) and oceanic region (Orme et al., 2005). The existing lineage diversity for *Plasmodium* and *Haemoproteus* spp. among the geographic regions exhibited similar diversity patterns to their avian hosts. However, *Haemoproteus* spp. diversity was significantly higher than that of *Plasmodium* spp. in all areas where the genera co-occurred, and *Haemoproteus* spp. were absent from the majority of oceanic regions whereas *Plasmodium* spp. were cosmopolitan. These differences are thought to be due to different vectors and the way they facilitate dispersion and colonization of avian Haemosporida in new bird communities (Clark et al., 2014). Furthermore, the spatial distribution of parasites is closely linked to the environmental, ecological, climatic and geographical conditions which influence the faunistic exchange between parasite populations (Ishtiaq et al., 2012).

The occurrence of haemosporidian parasites primarily relies on the presence of both an appropriate host and a competent vector (Apanius et al., 2000). *Plasmodium* spp. are transmitted by several mosquito species (Culicidae), the vectors for *Haemoproteus* parasites comprise hippoboscid and ceratopogonid flies and *Leucocytozoon* taxa are transmitted by simuliid and ceratopogonid flies (Santiago-Alarcon et al., 2012). However, there is only proof of one *Leucocytozoon* sp. (*Leucocytozoon caulleryi*) using ceratopogonid flies as vectors. It is believed that some *Leucocytozoon* spp.

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers MF442537–MF442624.

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might use ceratopogonid flies for their transmission in regions where the usual vector, the simuliid fly, is absent (Santiago-Alarcon et al., 2012). Parasites either have specialized host associations i.e. they tend to have a restricted range of hosts (intermediate host or vector) or they can use a broad spectrum of hosts i.e. they are generalists. To determine whether parasites are specialists or generalists is crucial in understanding parasite occurrence and distribution. Avian haemosporidian parasites present an interesting system for studying the hostparasitism strategies (specialization or generalism) of closely related parasites due to their high diversity and the diverse host fauna available in any particular geographical location (Beadell et al., 2009). However, to estimate ecological specialization is rather difficult. Different indices were tested in previous studies to quantify host specialization (Poisot et al., 2012). A very commonly used index is the STD (specificity taxonomic distinctness) index (Poulin and Mouillot, 2003) and variations of it (Hellgren et al., 2009). This index takes into account the average taxonomic or phylogenetic distance between pairs of host species used by a parasite. However, this index does not consider the frequency with which parasites are found in different host species. Exceptions in which birds are infected, which would not normally be a natural host, are as important in this index as natural infections and therefore the degree of specialization may be misjudged. Another estimator recently used in bird malaria research is Rao's QE (quadratic entropy) index (Ellis et al., 2015) where the parasites' host-breadth is calculated, taking into account the frequency of parasitism, but ignoring the taxonomy of hosts completely. However, host specificity of a parasite is not merely a function of how many host species it can exploit, but also of how closely related these host species are to each other (Poulin and Mouillot, 2003). Both indices therefore achieve unsatisfactory results. Another problem in determining the degree of host specialization is the limited data available. Therefore, in most of the previous studies only the specialization of the parasite genera *Plasmodium* and *Haemoproteus* were characterized (e.g. Bensch et al., 2000; Ricklefs et al., 2004; Okanga et al., 2014). However, the constant growth of the MalAvi database (Bensch et al., 2009) has made it increasingly easy to compare data on haemosporidian parasite lineages and enables us to validate analyses on parasites species or lineage levels as well. However, there is still insufficient data about suitable vectors, especially of species of the genera *Haemoproteus* and *Leucocytozoon,* to reliably link obvious host specialization with the associated vector and its host preference.

Oceanic archipelagos have been used as natural laboratories for understanding the evolutionary processes of speciation and divergence (Mayr, 1942; MacArthur and Wilson, 1967) and provide a unique opportunity to study community assemblages in similar yet geographically isolated units. The island of Madagascar is located approximately 400 km east of Africa in the Indian Ocean. It is classified as an important biodiversity hotspot (Myers et al., 2000) with over 359 bird species, of which more than half of Madagascar's breeding birds are endemic ($n = 199$). Given the documented and suspected threats posed by avian blood parasites to other island birds (Beadell et al., 2006; Matson and Beadell, 2010) haemosporidian parasites remain surprisingly understudied on Madagascar. During recent years, six studies have identified molecularly the prevalence and diversity of blood parasite lineages infecting wild birds from Madagascar and the neighboring Mascarenes (Barraclough et al., 2008a,b; Cornuault et al., 2012; Ishtiaq et al., 2012; Martínez-de la Puente et al., 2017; Schmid et al., 2017a; Ivanova et al., 2018), providing additional information to the previous knowledge based on blood smears (Barraclough et al., 2008a,b; Savage et al., 2009). However, the diversity and distribution of these parasites are poorly known compared with those of the neighboring African continent (e.g. Bensch et al., 2000; Beadell et al., 2009). Also, the review of Clark et al. (2014) does not include Madagascar. Therefore, it remains unclear whether Madagascar acts as an "oceanic region" such as the Hawaiian Islands and New Zealand with a low amount of haemosporidian parasite lineages present or whether it is not only a biodiversity hotspot for birds but also for haemosporidian parasites. Due to the vicinity to southern Africa, which has been characterized as a region with a high diversity of avian parasites of the Order Haemosporida, it is possible that a constant exchange of avian malarial lineages occurs through migrating birds and that the parasite diversity is similar to that of southern Africa. However, given the long-term geographical isolation and concurrent evolution of a unique avifauna with few migrants, the evolution of indigenous parasite fauna with endemic hosts could also be possible (Ishtiaq et al., 2012).

In this study, using mitochondrial DNA sequences (cytochrome *b* gene), we investigated the biodiversity of haemosporidian parasites in eastern Madagascar. Specifically, we aimed to (i) determine whether Madagascar represents a biodiversity hotspot for avian haemosporidian parasites; (ii) characterize the phylogenetic relationships of detected haemosporidian parasites and (iii) identify the kind of host specialization of the parasites.

2. Materials and methods

2.1. Site selection and characterization

Fieldwork was done in the months September - January, with the majority of samples taken in November and December (2003–2007, 2010, 2012, 2014 and 2016). Birds were sampled in the Maromizaha rainforest located in the eastern part of Madagascar (18°56′49″S, 48°27′33″E), 30 km from Moramanga city, at an altitude between 943–1213 m. Maromizaha harbours a unique community of highland and lowland species: 13 lemur, 20 reptilian, 60 amphibian (Giacoma et al., 2012) and 86 bird species (F. Woog, unpublished data) have been discovered so far. The area is protected and part of the Mantadia-Maromizaha-Zahamena rainforest corridor, a terrain that consists of hills with mountain ridges, valleys and small streams, dense and humid evergreen forest covers.

2.2. Field method

A total of 1067 birds (Supplementary Table S1) were caught in mistnets, subsequently sexed, aged, measured, weighed, ringed and a blood sample was obtained by puncture of the brachial vein before releasing the bird back to the wild. The protocol was approved by the Direction de la Préservation de la Biodiversité, Antananarivo, Madagascar. Blood was immediately stored in lysis buffer (Wink, 2006). This resulted in samples from 55 bird species belonging to 26 families, mostly of the Order Passeriformes. The most abundant bird species are shown in Table 1.

2.3. DNA extraction and parasite screening using PCR

Parasite DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. DNA was stored at −20 °C until further use. To prevent contamination with target DNA, a negative control (nuclease-free water) was included in each test run as well as a positive control (*Plasmodium berghei* DNA). The target sequence for amplification was a 1224 bp fragment of the haemosporidian mitochondrial cytochrome *b* gene. The PCR was conducted in two steps: the primer pair CytF1 (5′-GCCTAGA CGTATTCCTGATTATCC-3′) and CytR1 (5′-CATAATTATAACCT TACGGTCTG-3′) (Schmid et al., 2017b) was used to amplify a

Table 1
Avifauna from the Maromizaha rainforest in Madagascar ($n > 30$). All bird species belong to the Passeriformes. Prevalence of parasites and molecular ID of haemosporidian lineages in avian hosts are reported.

Family	Host species	*n*	*P* positive *n* (%)	*H* positive *n* (%)	*L* positive *n* (%)	Total positive *n* (%)	Lineages detected
Acrocephalidae	Madagascar Brush Warbler (*Nesillas typica*)	61	15 (25)	19 (31)	1 (2)	35 (57)	GRW04b, GRW09b, FOUMAD03, HYPMA01, NETYP01/03, ACNEW01, COLL7, FOMAD01
Bernieridae	Long-billed Greenbul (*Bernieria madagascariensis*)	31	6 (19)	3 (10)	2 (6)	10 (32)	GRW09b, FOUMAD03, BUL07, BERMAD02/03, BERZOS01, FOMAD01
	Spectacled Greenbul (*Xanthomixis zosterops*)	42	15 (36)	8 (19)	0	23 (55)	GRW04b, FOUMAD03, HYPMA01, BERZOS01-03
Hirundinidae	Madagascar Paradise Flycatcher (*Terpsiphone mutata*)	33	2 (6)	0	0	2 (6)	TERMUT01
Motacillidae	Madagascar Magpie Robin (*Copsychus albospecularis*)	37	10 (27)	11 (30)	0	21 (57)	GRW04b, FOUMAD03, COPALB02, TERMUT01, SFC3
Muscicapidae	African Stonechat (*Saxicola torquatus*)	46	18 (39)	0	0	18 (39)	GRW04b, GRW09b, FOUMAD03, SATOR01
Nectariniidae	Souimanga Sunbird (*Cinnyris sovimanga*)	38	4 (11)	22 (58)	1 (3)	27 (71)	GRW09b, FOUMAD03, CINCOQ01a, NENOT04b, NESOU02
Philepittidae	Velvet Asity (*Philepitta castanea*)	39	6 (15)	1 (3)	4 (10)	11 (28)	GRW04b, GRW09a/b, ZOSMAD01b, HYPMA05, PHICA01
Ploceidae	Forest Fody (*Foudia omissa*)	232	86 (37)	60 (26)	8 (3)	151 (65)	GRW04a/b, COLL7, GRW09b, FOUMAD02a/b/03, FOMAD01/02, BERMAD02, NESOU01, WW3, COLL4A, RBQ11b/c, FOUOM01, HYPMA04/05, NEWAM03
	Nelicourvi Weaver (*Ploceus nelicourvi*)	45	34 (76)	4 (9)	0	38 (84)	GRW04b, GRW09b, FOUMAD03, NESOU01, PLOSAK01, PLONE01
	Madagascar Fody (*Foudia madagascariensis*)	50	20 (40)	11 (22)	0	28 (56)	GRW04b, COLL7, GRW09b, FOUMAD02b/03, FOMAD02, RBQ11a/c
Pycnonotidae	Madagascar Bulbul (*Hypsipetes madagascariensis*)	98	38 (39)	17 (17)	22 (22)	69 (70)	GRW04b, GRW09b, BUL07, HYPMA01-05, NESOU01, ZOSMAD01a, BUL2, FOMAD01
Zosteropidae	Madagascar White-Eye (*Zosterops maderaspatanus*)	118	39 (33)	74 (63)	10 (8)	104 (88)	GRW04a/b, GRW09b, HYPMA01/04, ZOSMAD01b, NETYP03, ZOSMAD02, ZOSSTE01, ZOMAD01/05

P, Plasmodium; H, Haemoproteus; L, Leucocytozoon.

1332 bp fragment in the first PCR, whereas the internal primer pair CytFN (5′-GCTTTAAATGGTTGGAATATG-3′) and CytRN (5′-GTTTGC TTGGGAGCTGTAATC-3′) (Schmid et al., 2017b) amplified a 1224 bp fragment in the second PCR. The reaction mixture consisted of 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl2, 20 pmol of each primer, 200 µM of each dNTP, 1.25 units Ampli-Taq (Applied Biosystems, Carlsbad, USA) and approximately 10–100 ng of DNA for the first PCR in a total volume of 50 µl. For the second PCR 2 µl of the amplification product of the first PCR was used as a DNA template. Both PCRs were performed for 40 cycles with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 1.5 min.

After amplification, 5 µl of the PCR products stained with GelRed™ (BIOTREND, Köln, Germany) were visualized on a 1.5% agarose gel. If a sample showed unclear bands or no amplification product, the PCR was repeated. The PCR system used in this study was designed to amplify the cytochrome *b* gene of *Plasmodium* and *Haemoproteus* spp. but we were also able to detect some samples containing *Leucocytozoon* DNA, presumably with a low sensitivity. Our results therefore underestimate the prevalence of *Leucocytozoon* spp.

Amplification products were then purified using the PCR Product Purification Kit (Roche, Mannheim, Germany) and after sequencing (GATC Biotech AG, Germany) the resulting sequences were checked and edited. The final sequences (1063 bp) were then distinguished by identifying their closest matches in GenBank (Benson et al., 2013) using the NCBI nucleotide BLAST search and a BLAST search of the MalAvi database (Bensch et al., 2009; as of January 2018). For sequences without clear chromatograms after repeating PCR and sequencing ($n = 76$) we only distinguished the parasite genus for the evaluation of prevalence.

Samples whose chromatograms showed distinct double peaks were suspected of containing multiple infections. By amplifying the 1063 bp fragment of the cytochrome *b* gene in two separate, overlapping fragments (“front” and “back”), using the PCR protocol of Schmid et al. (2017b), an attempt was made to identify those multiple infections. Due to the different sensitivity of the primer pairs used, it was possible to amplify the sequence of different parasites in the “front” and in the “back”. The comparison of those sequences with sequences already isolated in this study enabled the identification of double infections.

As our fragment is more than double the length compared with that used in most of the previous studies, we found only three published sequences which were 100% identical with regard to the full length of our 1063 bp fragment. All resulting lineages in our study were deposited in GenBank (accession numbers MF442537–MF442624). Based on the different fragment sizes used in MalAvi and GenBank, the nomenclature of lineages differs (Supplementary Table S2). But in the following text only the nomenclature of MalAvi is used.

2.4. Phylogenetic analysis

A total of four phylogenetic trees were created in this study. The dataset used for the first phylogenetic reconstruction consisted of haemosporidian lineages obtained in this study and reference sequences of different haemosporidian species downloaded from GenBank, each trimmed to 1063 bp to ensure consistency in sequence length. A cytochrome b sequence from *Theileria annulata* (KF732030.1) was used as an outgroup. The second tree included only the *Plasmodium* lineages isolated in this study and the third all *Haemoproteus* spp.. For both trees, the lineage *Leucocytozoon* sp. FOMAD01 was used as an outgroup. The dataset of the fourth tree contained all *Leucocytozoon* lineages obtained in this study and the lineage *Plasmodium* sp. GRW04b as an outgroup. The different datasets were analyzed in MrModeltest v2.3 (Nylander, 2004) to determine which nucleotide substitution model was appropriate based on the Akaike Information Criterion (Huelsenbeck and Crandall, 1997). Phylogenetic analyses were performed using Bayesian inference performed in MrBayes v3.2.6

(Ronquist and Huelsenbeck, 2003). The appropriate model was implemented and two Markov chains were run simultaneously for 50 million generations; trees were sampled every 1000 generations, resulting in 50,000 trees. Twenty-five percent of the trees were discarded as "burn-in". The remaining trees were used to construct a majority rule consensus tree and to calculate posterior probabilities. To estimate bootstrap values and to check for congruence of phylogenetic relationships across multiple approaches, a maximum likelihood (ML) approach was implemented in MEGA v7 (Tamura et al., 2013) for each tree. Phylogenies were generated by implementing the best fitting model using 1000 replicates. The Bayesian majority consensus tree and ML phylogram were viewed and edited with FigTree v1.4.3 (Andrew Rambaut, University of Edinburgh, England) and MEGA v7. The sequence divergence between different lineages and specified groups was calculated using the Jukes-Cantor model of substitution implemented in the program MEGA v7. In cases of sequence divergence of 5% or more, lineages or groups were considered to be morphologically distinct species (Hellgren et al., 2007a).

2.5. Host diversity and specialization

In order to draw conclusions about a possible specialization of the parasite species, the host diversity index was established. This index is based on the haplotype diversity index by Nei (1987). The index is calculated as follows:

$$Hd = n(1 - \Sigma xi^2)/(n - 1)$$

where n is the total number of birds the considered parasite species was isolated from and xi is the frequency of a given bird species/genus/family in the population. Depending on the taxonomic level that is considered, the index is then called HdS (bird species), HdG (bird genus) or HdF (bird family). The calculated host diversity can be used as an indicator for the specialization of the considered parasite species. A value of 1 represents a maximum of host diversity and 0 a minimum of host diversity. Generalist parasite species are considered to be capable of infecting many different host taxa, whereas specialists have a limited range of potential host taxa. Therefore, parasite species showing a high Hd (>0.6) were considered generalists and those with a value close to 0 (<0.3) as specialists. The Hd values were calculated for every parasite species i.e. group and for each parasite genus. Means are presented ±1 S.D. We tried to obtain more evidence on the degree of specialization of each parasite group or lineage by including the information on homologous sequences from the MalAvi database (as of January 2018). The homologous sequences of the MalAvi database are only 479 bp long and thus may not represent identical lineages with regard to the cytochrome *b* sequence we used (1063 bp). Nevertheless, potential differences are considered in order to represent different haplotypes of the same parasite species.

2.6. Avian haemosporidian hotspot categories

To decide whether Madagascar represents a hotspot for avian haemosporidian parasites or not, a comparison was made regarding how many parasite lineages a single bird is parasitized by, on average. Based on the regions described by Clark et al. (2014), values for avian or haemosporidian hotspot, non-hotspot and the special non-hotspot "oceanic region" were calculated using only community studies and an average was created for each region.

3. Results

3.1. Prevalences of haemosporidian parasites (genera and lineages)

Using a PCR-based approach, a total of 1067 avian blood samples of 55 species from the Maromizaha rainforest (Madagascar) were screened and in 649 individuals (60.8%), haemosporidian DNA was detected (Supplementary Table S1). Out of these samples, 343 (52.9%) harboured *Plasmodium* and 287 (44.2%) *Haemoproteus* DNA. As an additional side-effect we detected 66 (10.2%) *Leucocytozoon* spp. infections. Furthermore, 47 samples (4.4% of 1067) contained at least double infections from parasites of the same or different genera, of which 38 double infections could be distinguished and determined to lineage level (Table2). Most double infections were detected in *Zosterops maderaspatanus* ($n = 19$), whereas *Hypsipetes madagascariensis* ($n = 8$) had the greatest variety of double infections.

Partial cyt*b* mtDNA sequencing revealed 88 genetically distinct haemosporidian lineages in the Malagasy bird population (Supplementary Table S2) with 29 *Plasmodium*, 42 *Haemoproteus* and 17 *Leucocytozoon* lineages. By far the most abundant *Plasmodium* lineage was GRW04b ($n = 99$); the two most abundant *Haemoproteus* lineages were ZOSMAD01a ($n = 44$) and FOUMAD02b ($n = 42$); and

Table 2
Variations of double infections (parasite lineages 1 and 2) detected in Malagasy birds.

Bird family	Bird species	Parasite 1	Parasite 2	*n*
Acrocephalidae	*Acrocephalus newtoni*	GRW09b	FOUMAD03	1
Bernieridae	*Bernieria madagascariensis*	BUL07	HYPMA05	1
Nectariniidae	*Cinnyris notatus*	COSUN2	NENOT05	1
Ploceidae	*Foudia omissa*	COLL7	GRW09b	1
		NESOU01	WW3	1
		COLL7	RBQ11c	1
	Foudia madagascariensis	COLL7	RBQ11c	3
Pycnonotidae	*Hypsipetes madagascariensis*	GRW04b	FOMAD01	1
		GRW04b	HYPMA05	1
		GRW09b	FOMAD01	1
		GRW09b	HYPMA05	1
		NESOU01	FOMAD01	1
		NESOU01	ZOSMAD01a	1
		FOMAD01	HYPMA05	1
		FOMAD01	HYPMA05	1
Zosteropidae	*Zosterops maderaspatanus*	GRW04b	ZOSMAD01a	9
		GRW04b	ZOSMAD01b	5
		GRW04b	ZOSMAD02	2
		ZOSMAD01a	HYPMA04	1
		ZOSMAD01a	ZOSSTE01	1
		ZOSMAD01b	NETYP03	1

the most abundant *Leucocytozoon* lineages were FOMAD01 (n = 16) and HYPMA05 (n = 12). According to the NCBI BLAST search in GenBank, only three of the detected lineages (GRW04a, COLL4a and GRW06) are 100% homologous to previously published sequences at full length; all other lineages represent novel cyt*b* lineages with regard to the full length of our 1063 bp fragment. The comparison of the sequences found with shorter sequences from MalAvi (463–479 bp) revealed in total 42 matches (Supplementary Table S2). Moreover, partial sequences of six lineages (BUL07, GRW04a/b, COLL7 and GRW09a/b) have been previously isolated from mosquito species on Madagascar (Schmid et al., 2017a; Supplementary Table S1).

3.2. Phylogeny of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* lineages

The phylogenetic tree containing all detected haemosporidian lineages from Madagascar together with previously published haemosporidians (Supplementary Fig. S1) revealed a clear delimitation of the Haemosporida from the outgroup *Theileria annulata* (KF732030.1) with strong nodal support. All lineages detected in this study formed monophyletic groups for each parasite genus. For all detected *Plasmodium* lineages, both Bayesian interference (Fig. 1) and ML predictions (tree not shown, but bootstrap values imported to Bayesian tree) suggest that the 29 lineages form 13 clades (Gp 1–13) with strong nodal support. A distance matrix (Supplementary Table S3) confirms that the average distance between the set groups is rather high (3.2–11.5%), while the lineages forming these groups are genetically quite similar (0.094–2.28%).

The phylogenetic tree of all 42 *Haemoproteus* lineages detected (Fig. 2) can be divided into 11 clades (Gp 1–11) with strong nodal support. The genetic distances between these groups are high (3.9–12.9%), but within the groups the genetic distance varies only between 0.13 and 2.89% (Supplementary Table S4). None of the detected *Haemoproteus* lineages was 100% identical with previously published sequences in GenBank over the full-length fragment. A comparison of the lineages with those from the MalAvi database revealed 14 sequences with 100% homology with previously published sequences (Supplementary Table S2). However, these sequences have a maximum length of 479 bp. Therefore, different haplotypes based on our 1063 bp fragment could have the same homologous sequence based on the 479 bp fragment. In these 17 cases, the genetic difference at full length is only 1or 2 bp and usually results in silent mutations (Supplementary Table S5). The only exceptions are the haplotypes RBQ11a, b and c. While lineages RBQ11a and RBQ11c have the same protein sequence, RBQ11b shows a mutation at position 304 which changes Leucine to Isoleucine.

All *Leucocytozoon* lineages detected in this study can be divided into five distinct, wellsupported clades (Groups 1–5) (Fig. 3). The

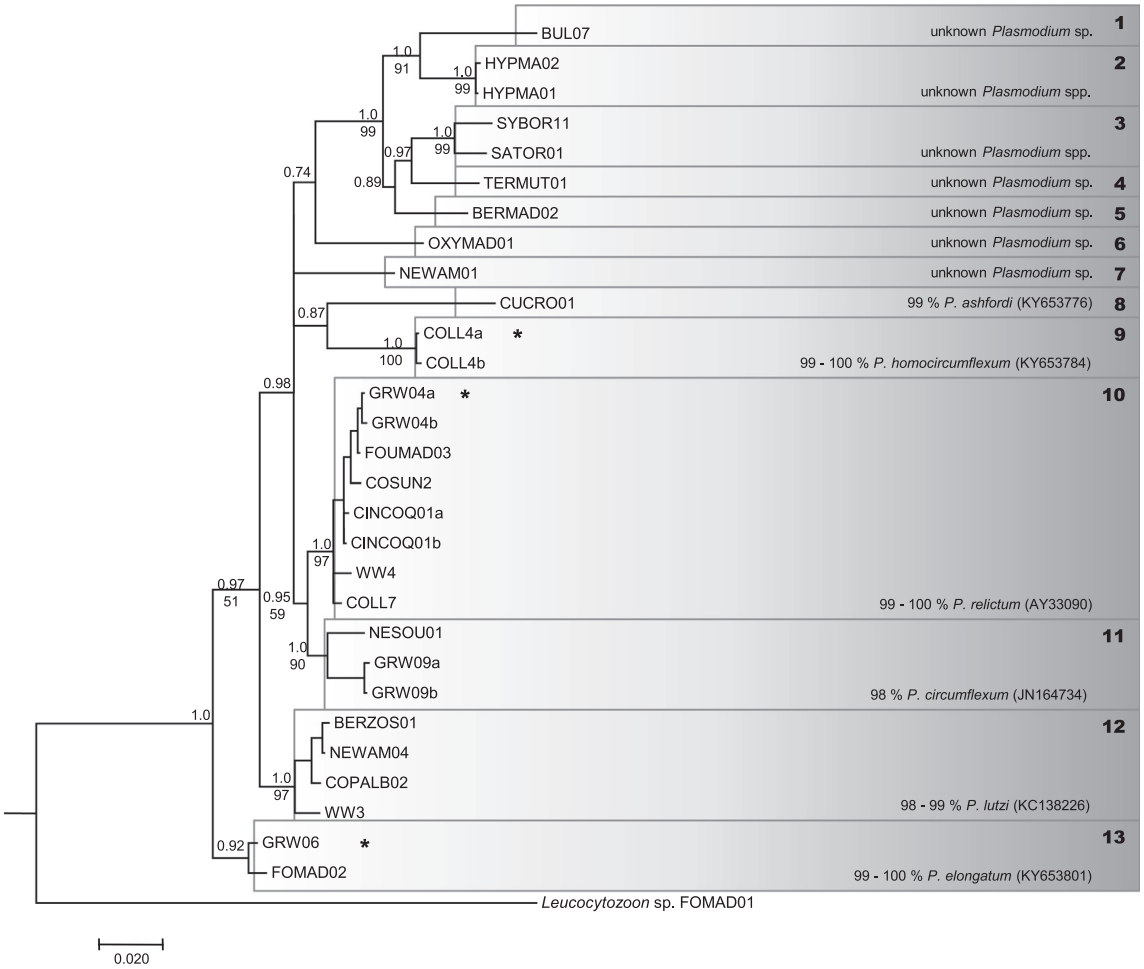

Fig. 1. Bayesian major consensus tree of *Plasmodium* lineages (1063 bp fragment of cytochrome *b*). The visualized phylogeny was constructed using Bayesian interference performed in MrBayes. The posterior probability is shown as the denominator on the branches. When the Bayesian major consensus tree was congruent with the constructed maximum-likelihood approach implemented in MEGA, the bootstrap support is shown as the nominator. Due to their genetic distances, lineages were divided into 13 groups, representing distinct species. The NCBI BLAST search match (homology, species name and GenBank accession number) for each group is shown and lineages with 100% identity are marked with an asterisk. Detailed information about BLAST search matches is available in Supplementary Table S1, Supporting information.

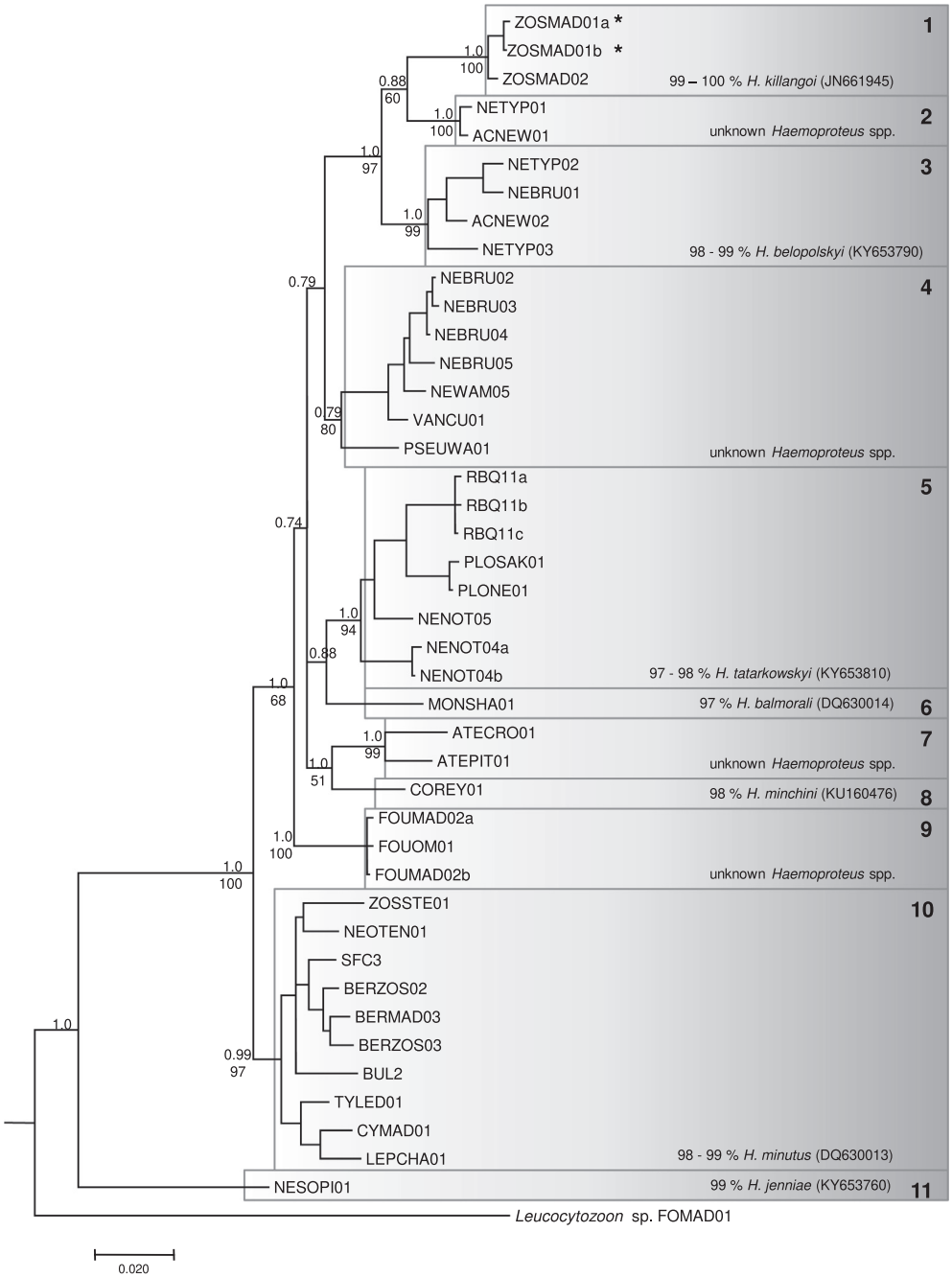

Fig. 2. Bayesian major consensus tree of *Haemoproteus* lineages (1063 bp fragment of cytochrome *b*). The visualized phylogeny was constructed using Bayesian interference performed in MrBayes. The posterior probability is shown as the denominator on the branches. When the Bayesian major consensus tree was congruent with the constructed maximum-likelihood approach implemented in MEGA, the bootstrap support is shown as the nominator. Due to their genetic distances, lineages were divided into 11groups, representing distinct species. The NCBI BLAST search match (homology, species name and GenBank accession number) for each group is shown and lineages with 100% identity are marked with an asterisk. Detailed information about BLAST search matches is available in Supplementary Table S1, Supporting information.

distance matrix (Supplementary Table S6) supports this classification with high genetic distances between the proposed groups (3.8–27.4%) and distances within the groups that range from 0.63 to 2.36%. The *Leucocytozoon* Group 5, isolated only from individuals of the Order Accipitriformes, had the highest mean between-group distances of all. Only the lineage RECOB3 isolated from *Hartlaubius auratus* (Passeriformes, Sturnidae) showed a 100% homology with a 607 bp sequence of *Leucocytozoon gentili* (DQ451435) previously isolated from an House Sparrow, *Passer domesticus* (Passeriformes, Passeridae), by Martinsen et al. (2006), published in GenBank. Performing a BLAST search in the MalAvi database revealed seven lineages with 100% homology (Supplementary Table S2). All these lineages have a length of only 476–478 bp and therefore do not cover even half of the *Leucocytozoon* fragment amplified in this study.

3.3. Different degrees of specialization for haemosporidian parasite lineages

Based on the results in which bird species the parasite lineages were found (Supplementary Tables S7–9), Hds were calculated for parasite groups with $n \geq 3$ (Supplementary Table S10). Indices for

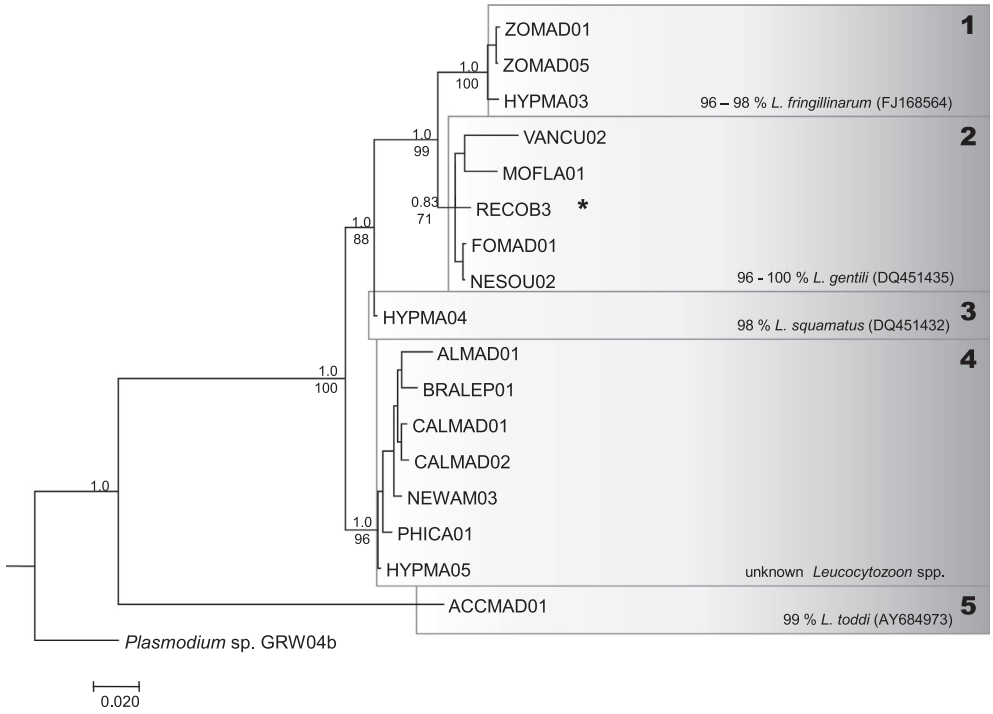

Fig. 3. Bayesian major consensus tree of *Leucocytozoon* lineages (1063 bp fragment of cytochrome *b*). The visualized phylogeny was constructed using Bayesian interference performed in MrBayes. The posterior probability is shown as the denominator on the branches. When the Bayesian major consensus tree was congruent with the constructed maximum-likelihood approach implemented in MEGA, the bootstrap support is shown as the nominator. Due to their genetic distances, lineages were divided into five groups, representing distinct species. The NCBI BLAST search match (homology, species name and GenBank accession number) for each group is shown and lineages with 100% identity are marked with an asterisk. Detailed information about BLAST search matches is available in Supplementary Table S1, Supporting information.

Plasmodium groups ($n = 11$) ranged between 0.24 and 1 (HdS = 0.72 ± 0.21; HdG = 0.65 ± 0.34; HdF = 0.62 ± 0.28), for *Haemoproteus* groups ($n = 8$) between 0 and 0.81 (HdS = 0.42 ± 0.28; HdG = 0.25 ± 0.30; HdF = 0.19 ± 0.28) and for *Leucocytozoon* groups ($n = 4$) between 0.2 and 0.84 (HdS = 0.61 ± 0.26; HdG = 0.61 ± 0.29; HdF = 0.6 ± 0.28). The parasite lineage COLL7 accounted for 18.95% of all *Plasmodium*-positive samples and was isolated only from birds of the Family Ploceidae. Therefore, the different Hds for this lineage were calculated separately (HdS = 0.35; HdG = 0.03; HdF = 0). Due to proposed subgroups (H5.1 and H5.2; H10.1-6) in *Haemoproteus* Group 5 and 10, both groups were divided and indices were calculated for each subgroup (Supplementary Table S8). Including these subgroups in the calculation of mean Hd for each parasite genus (Fig. 4) resulted in even lower values for *Haemoproteus* (HdS = 0.35 ± 0.32; HdG = 0.19 ± 0.28; HdF = 0.02 ± 0.04; $n = 12$), indicating a higher degree of specialization in these subgroups. The proposed specialization of each parasite group was distinguished based on our results and on records from previous studies (Table 3).

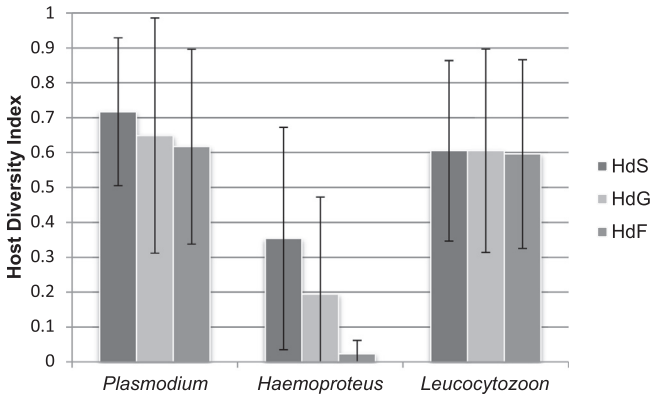

Fig. 4. Mean host diversity indices for each haemosporidian genus ± S.D. using Hd values of parasite groups. Depending on the taxonomic level, the index is called HdS (bird species), HdG (bird genus) or HdF (bird family). It is assumed that the calculated host diversity is an indicator for the specialization of the parasite species. A value of 1 represents a maximum of host diversity and 0 a minimum of host diversity. The value of each parasite group is available in Supplementary Table S7, Supporting information.

4. Discussion

With a total of 1067 blood samples from 55 bird species, our study resulted in the first large-scale description of avian malaria in Madagascar based on molecular approaches. We detected a high prevalence of 60.8% for haemosporidian parasites. The genera *Plasmodium* (52.9%) and *Haemoproteus* (44.2%) showed much higher prevalences than previously reported for Madagascar. This may be due to the much smaller sample sizes of those studies (i.e. $n = 72$; Ivanova et al. (2018)). In a study of islands in the western Indian Ocean ($n = 150$), including Madagascar ($n = 28$), an overall prevalence of *Plasmodium* spp. and *Haemoproteus* spp. of 45.3% was registered (Ishtiaq et al., 2012). The highest prevalence in a community study was reported by Lutz et al. (2015) from Malawi (East Africa) examining 532 individuals of 152 bird species with a prevalence of 79.1% for avian haemosporidians representing 248 different lineages. Assuming that the prevalence of *Leucocytozoon* spp. (6%) we detected is an underestimation, the prevalence of all haemosporidian genera in Madagascar may be comparable with that found in Malawi. As postulated by Clark et al. (2014), avian haemosporidians seem to exhibit similar diversity patterns to their avian hosts. In regions defined as avian hotspots, one bird species harbors an average of 1.24 parasite lineages (Beadell et al., 2004; Silva-Iturriza et al., 2012; Lutz et al., 2015; Mata et al., 2015) of

Table 3
Proposed specialization of each haemosporidian parasite group on Madagascar compared with proposed specialization based on previously published data in the MalAvi database. Specialists on bird species, genus or family level are marked in bold and the predicted hosts are shown. '-' indicates that no distinction between generalist and specialist can be made due to a lack of data.

Parasite			Specialization		
Genus	Group	Lineage	MalAvi	Madagascar	Host
Plasmodium	1	BUL07	Generalist	**Specialist**	*Hypsipetes madagascariensis*
	2	HYPMA01 and HYPMA02	–	Generalist	
	3	SYBOR11 and SATOR01	–	Generalist	
	4	TERMUT01	–	Generalist	
	5	BERMAD02	–	Generalist	
	6	OXYMAD01	–	–	
	7	NEWAM01	–	**Specialist**	*Newtonia* spp.
	8	CUCRO01	-	–	
	9	COLL4a/b	Generalist	Generalist	
	10	GRW04a/b, FOUMAD03, COSUN2, CINCOQ01a/b, WW4 and COLL7	Generalist	Generalist	
		COLL7	Generalist	**Specialist**	Ploceidae
		COSUN2	–	**Specialist**	*Cinnyris* spp.
		WW4	–	**Specialist**	Cisticolidae
	11	GRW09a/b and NESOU01	Generalist	Generalist	
	12	BERZOS01, NEWAM04, COPALB02 and WW3	Generalist	Generalist	
	13	GRW06 andFOMAD02	Generalist	Generalist	
Haemoproteus	1	ZOSMAD01a/b and ZOSMAD02	Specialist	Specialist	*Zosterops* spp.
	2	NETYP01andACNEW01	–	Specialist	Acrocephalidae
	3	NETYP02, NEBRU01, ACNEW02 and NETYP03	–	Specialist	Acrocephalidae
	4	NEBRU02 – 05, NEWAM05, VANCU01 and PSEUWA01	–	Specialist	Vangidae
	5.1	RBQ11a/b/c, PLOSAK01 and PLONE01	Specialist	Specialist	Ploceidae
	5.2	NENOT04a/b and NENOT05	–	Specialist	*Cinnyris* spp.
	6	MONSHA01	–	–	
	7	ATECRO01 and ATEPIT01	–	Specialist	*Atelornis* spp.
	8	COREY01	–	–	
	9	FOUMAD02a/b and FOUOM01	–	Specialist	*Foudia* spp.
	10.1	ZOSSTE01	Specialist	Specialist	Zosteropidae
	10.2	NEOTEN01	–	–	
	10.3	SFC3	Specialist	Specialist	Muscicapidae
	10.4	BERZOS02, BERMAD03 andBERZOS03	–	Specialist	Bernieridae
	10.5	BUL2	Specialist	Specialist	Pycnonotidae
	10.6	TYLED01, CYMAD01andLEPCHA01	–	Specialist	Vangidae
	11	NESOPI01	–	–	
Leucocytozoon	1	ZOMAD01, ZOMAD05andHYPMA03	Specialist	**Specialist**	*Zosterops* spp.
	2	VANCU02, MOFLA01, RECOB3, FOMAD01 and NESOU02	Generalist	Generalist	
	3	HYPMA04	–	Generalist	
	4	ALMAD01, BRALEP01, CALMAD01, CALMAD02, NEWAM03, PHICA01 and HYPMA05	–	Generalist	
	5	ACCMAD01	–	Specialist	*Accipiter* spp.

the genera *Plasmodium* and/or *Haemoproteus* (Supplementary Table S11). In regions defined as non-hotspots, on average only 0.9 lineages were found in each host (Dimitrov et al., 2010; Astudillo et al., 2013; Santiago-Alarcon et al., 2016; Smith et al., 2018) and in oceanic regions 0.34 (Hellgren et al., 2011; Howe et al., 2012). In our study one bird species harbors an average of 1.29 parasite species, suggesting that according to the definition by Clark et al. (2014), Madagascar is a hotspot for avian haemosporidian parasites. In contrast to other oceanic regions such as New Zealand, the Hawaiian Islands or the Marquesas where *Haemoproteus* spp. seem to be absent (Clark et al., 2014), a high number of *Haemoproteus* lineages ($n = 42$) exists on Madagascar. This could be due to the closeness of Madagascar to Africa. Since Madagascar is not as isolated as the other islands mentioned above, natural colonization of *Haemoproteus* spp. from mainland Africa has probably been possible without anthropogenic introductions of infected hosts and/or suitable vectors. An additional explanation could be the pylogeographically older age of Madagascar. Parts of the vector-parasitesystem may have been already present before the separation from mainland India.

Clark et al. (2014) also observed that *Haemoproteus* spp. diversity was significantly higher than that of *Plasmodium* spp. in all areas where the genera co-occurred. This is consistent with our results if focusing on lineage numbers only. Of the genus *Plasmodium* we detected 29 distinct lineages; of *Haemoproteus* spp. more than 40. Based on the genetic differences, we suspect that these groups might represent distinct species. However, it cannot be ruled out that some lineages in the groups are cryptic species, thus we may underestimate species richness. Future studies should also consider morphological nuclear sequence data.

A comparison of the *Plasmodium* lineages with sequences available in GenBank revealed that Groups 8–13 might represent distinct *Plasmodium* spp. that have already been described genetically and morphologically (*Plasmodium ashfordi, Plasmodium homocircumflexum, Plasmodium relictum, Plasmodium circumflexum, Plasmodium lutzi* and *Plasmodium elongatum*). All other groups might represent unknown *Plasmodium* spp. (Groups 4, 5, 6) or species for which a partial sequence of the cytochrome *b* gene is available on MalAvi database (Groups 1, 2, 3, 7) but no assignment to any described species has yet been given. Although more *Haemoproteus* lineages were found, these cluster in fewer groups in contrast to *Plasmodium* (*Plasmodium*: 13 groups i.e. predicted species; *Haemoproteus*: 11 groups). Therefore, the biodiversity of haplotypes seems to be higher for *Haemoproteus* ($n = 42$) in comparison

with *Plasmodium* ($n = 29$), while biodiversity at the species level seems to be equal or even higher for *Plasmodium* spp. However, in all studies dealing with avian haemosporidian parasite detection using PCR methods, only primers have been used that detect both *Plasmodium* and *Haemoproteus* spp. Due to the high sequence similarity of the two genera, there are no genus-specific primers to date. All recorded prevalences for *Plasmodium* or *Haemoproteus* infections of these studies might therefore be affected by differences in the specificity and sensitivity of the PCR used.

The newly used Hd index for different taxonomic host levels takes into account the frequency at which parasites were found in host species together with the taxonomy of bird hosts. For the genus *Plasmodium* the mean Hd values (HdS = 0.72, HdG = 0.65, HdF = 0.62) indicate that this genus is a generalist with regard to all taxonomic levels, which is consistent with previous publications (e.g. Beadell et al., 2009; Cumming et al., 2013; Okanga et al., 2014).

Within the *P. relictum* complex (Group 10), we detected possible specialization of some lineages. COLL7 was the second most abundant lineage ($n = 65$) and was only isolated from the Ploceidae (HdF = 0), suggesting a specialization on this bird family (Table 3). Its previously described homologous pendant, lineage COLL7 (DQ368376), has been isolated from six different bird families in Africa and migratory *Ficedula* spp. (Muscicapidae) from Sweden and Hungary which winter in those African regions. Three of five described host families in Africa (Nectariniidae, Ploceidae, Pycnonotidae) were sampled frequently in this study. However, only individuals of the family Ploceidae were infected. Therefore, we assume that the lineage COLL7 is a generalist in mainland Africa, but may be a specialist in Madagascar. The endemic bird species within the Pycnonotidae (*Hypsipetes madagascariensis*) and Nectariniidae (*Cinnyris notata* and *Cinnyris sovimanga*) could have developed resistance to the lineage COLL7 over the period of their isolation. The observed specialization might also be a consequence of different vector mosquitoes responsible for transmission. The proposed vector mosquito of this lineage found on Madagascar is an *Uranotaenia* sp. (Schmid et al., 2017a). Other lineages of the *P. relictum* complex for which we postulate host specificity are COSUN2, CINCOQ01a/b and WW4. We isolated all lineages only a few times from individuals belonging to the Nectariniidae (COSUN2 and CINCOQ01a/b) and Cisticolidae (CINCOQ01b and WW4). COSUN2 (DQ847269) has been isolated only from *Cinnyris* spp. (Nectariniidae) in Nigeria (Hellgren et al., 2007b). CINCOQ01a and bare both 100% homologous to CINCOQ01 (DQ659560) which was isolated from *Cinnyris* spp. in Madagascar and Mayotte (Beadell et al., 2006; Ishtiaq et al., 2012). The lineage WW4 (AF495578) has been previously found multiple times in birds of four different families. The lineage was found either in Malawi (Africa) and mainly in Cisticolidae (Lutz et al., 2015) or in European migratory birds, all of which winter in Africa. These results lead to the hypothesis that this lineage could be a specialist in its endemic area (Africa). However, the implication of European migratory birds makes the assessment about specialization more difficult.

The *Plasmodium* Group 1 (BUL07) showed a very low Hd value of 0.242 at all taxonomic levels, indicating that this parasite has a narrow host range. BUL07 (EU810628) has been isolated in a previous study from 33 bird species belonging to 23 families in Malawi (Africa) alone (Lutz et al., 2015). Representatives of seven of the mentioned families are also present in Madagascar but we isolated the lineage almost exclusively from *Hypsipetes madagascariensis* (Pycnonotidae). This might indicate that the lineage represents a generalist but on Madagascar it acts as a specialist since the representatives of other bird families there seem not to be suitable hosts. Another exception could be Group 7 (NEWAM01). This lineage was isolated only four times from two different *Newtonia* spp. (Vangidae). Lineage NEWAM01 (KX506750) has been previously isolated just from a single individual of *Newtonia amphicroa* in Madagascar (Ivanova et al., 2018). These findings suggest that the lineage might be restricted to Madagascar and is specialized in birds of the genus *Newtonia* which needs to be confirmed by a larger sample size.

The assumption that all *Plasmodium* spp. are generalists is not supported by our data. Rather, a lot of factors need to be considered when assessing specialization. For example, geographic isolation could lead to regional differences in the specialization of parasites. Additionally, it is necessary to further examine the role of migratory birds in terms of specialization as they seem to sometimes distort assessments. Jenkins et al. (2011) already suggested that as migration is an energetically costly activity, resources may be tradedoff from immune defense, making it likely that migrants are more susceptible to avian malarial parasites than resident birds. Therefore, specialized parasite lineages can also be found in migrants although resident representatives of the same genus or family are in fact not suitable hosts. Further, it is possible that abortive infections were detected by the method used. In these cases, the initial parasite development occurs (tissue stages in birds develop), but the parasites are not able to develop gametocytes and therefore are not able to complete their life cycles (Valkiunas, 2005; Palinauskas et al., 2013). Because DNA from such haemosporidian tissue stages may leak into the blood, it is possible that the reported host ranges of only molecular-based studies is overestimating the ranges of their competent hosts (Chagas et al., 2017) as might be the case for migratory birds.

Hd values between 0 and 0.81 for *Haemoproteus* groups indicate that they are specialists – especially at the family level of bird hosts. These observations coincide with previous reports of homologous sequences. The *Haemoproteus* Group 1 seems to consist of three haplotypes of the species *Haemoproteus killangoi*. ZOSMAD01a and b which have been isolated mainly from *Zosterops maderaspatanus* (68 out of 70) are 100% homologous to the previously detected lineage ZOSMAD01 (JN661945). According to the MalAvi database, this lineage has been found at least 32 times in other *Zosterops* spp. in Africa, which suggests a specialization of the parasite. Single findings of this parasite in birds of other families could be due to a weakness of the immune system or co-infections (Sorci, 2013).

The lineage BUL2 was detected exclusively in 16 samples of *Hypsipetes madagascariensis* (Pycnonotidae) and therefore is considered a specialist. The homologous sequence BUL2 (DQ847195) was, according to the MalAvi database, previously isolated from four bird species of the family Pycnonotidae in Africa and Israel, and one *Phylloscopus trochilus* (Sylviidae) from Nigeria. These findings support our hypothesis that BUL2 is specialized on Pycnonotidae, although the migratory bird *P. trochilus* seems to represent an exception. The lineage PLOSAK01 was detected twice in *Ploceus nelicourvi* in this study and was previously isolated from two individuals of *Ploceus sakalava* on Madagascar (Ishtiaq et al., 2012). These two species are both endemic in Madagascar with *P. nelicourvi* being present in eastern Madagascar and *P. sakalava* in western Madagascar. This lineage may be specialized on *Ploceus* spp. and may have evolved only on Madagascar and the Mascarenes. The three lineages RBQ11a, b and c were isolated mainly from *Foudia* taxa (Ploceidae) and are all 100% identical with the previously published lineage RBQ11 (EF117229) that has been exclusively isolated from various bird species of the family Ploceidae in Africa, which may therefore indicate specialization on African Ploceidae. None of our lineages forming the *Haemoproteus* Group 4 have been previously described and we suggest a new *Haemoproteus* sp. that is specialized on Vangidae that are mostly endemic to Madagascar. In a study focusing on the morphological identification of

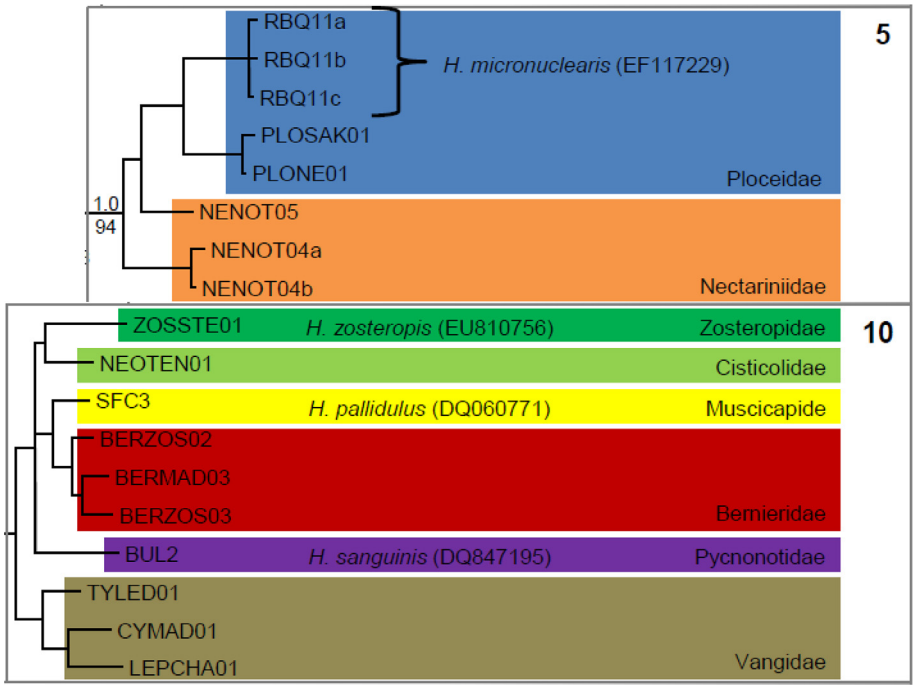

Fig. 5. *Haemoproteus* groups 5 and 10 are shown as an excerpt from the *Haemoproteus* phylogeny. Both groups are divided into subgroups according to matches with 100% homologous partial sequences available in the MalAvi database (species name and GenBank accession number) and proposed specialization at the bird family level. The bird families are marked with different colours.

haemosporidian parasites in blood smears of vangas, two new species of *Haemoproteus* were described (Savage and Greiner, 2004). *Haemoproteus* Group 4 might represent one of these described species.

The lineage ZOSSTE01 was isolated twice from *Z. maderaspatanus* (Zosteropidae) in this study. ZOSSTE01 (EU810756) is linked to the morphospecies *Haemoproteus zosteropis*. To date it has been exclusively found in the Zosteropidae in all regions where this bird family is distributed (Valkiunas, 2005), which suggests a strong specialization of this parasite.

Due to their genetic distances (>5%) from all other postulated groups, the *Haemoproteus* Groups 5 and 10 may actually consist of more than two species (Fig. 5). Group 5 might in fact represent two species: one of them, *Haemoproteus micronuclearis,* specialized on Ploceidae, the other one might be an unknown *Haemoproteus* sp. specialized on Nectariniidae. The predicted *Haemoproteus* Group 10 might even consist of five different species, all specialized on different families. An identification of these parasites in blood smears and data on nuclear markers might support this predicted classification and could show whether these are possibly cryptic species.

To date, there is no definitive statement about the specialization of *Leucocytozoon* spp. *Leucocytozoon* is often neglected in studies of avian malaria and therefore less information is available compared with *Plasmodium* or *Haemoproteus*. The Hd values calculated in our study, especially those of Groups 2 and 4, indicate that *Leucocytozoon taxa* might be generalists. The lineage RECOB3 (DQ847221), found in *Hartlaubius auratus* (Sturnidae), has already been detected in nine different bird families, indicating that this lineage may be a generalist. Another example for a putative generalist parasite lineage is FOMAD01 which was detected in 16 samples from birds of five different families (Pycnonotidae, Bernieridae, Acrocephalidae, Ploceidae and Nectariniidae). FOMAD01 (JN032605) has also been isolated from birds of the families Pycnonotidae, Ploceidae and Nectariniidae (Cornuault et al., 2012). As the lineage was only found in Madagascar, the Comoros and the Mascarenes to date, it might be restricted to this area due to a specialization on its vector.

However, the *Leucocytozoon* Group 1 seems to be an example of a specialized species of the genus *Leucocytozoon.* ZOMAD01 (JN032614) and ZOMAD05 (JN032621) have been isolated nine times from *Z. maderaspatanus* in this study. HYPMA03 was found to be 99% homologous with ZOBOR03 (JN032613). All these published sequences were previously isolated from *Zosterops* spp. endemic to Madagascar, the Comoros and the Mascarenes (Cornuault et al., 2012). The latter already suggested that their detected clade B, which contains ZOMAD01, ZOMAD05 and ZOBOR03, shows a high specificity to *Zosterops* and is restricted to Madagascar and surroundings. Our findings support this hypothesis. This example clearly shows that even for *Leucocytozoon,* no general statement about specialization can be made.

Madagascar indeed represents a biodiversity hotspot for avian malaria parasites, as we detected 71 genetically distinct parasite lineages with 1.29 lineages of *Plasmodium* and *Haemoproteus* per bird species. We suspect that the detected haemosporidian parasite lineages represent at least 29 groups i.e. proposed species. Our results strongly support previous works demonstrating the elastic host ranges of avian haemosporidians. In general, *Plasmodium* and *Leucocytozoon* spp. seem to be generalists, whereas *Haemoproteus* spp. might be specialists. However, as demonstrated in various examples, single parasite species or lineages can deviate from this rule and should be examined individually.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.11.001.

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