

Article

Avian Haemosporidian Diversity on Sardinia: A First General Assessment for the Insular Mediterranean

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Abstract: The Western Palearctic is one of the most investigated regions for avian haemosporidian parasites (*Haemoproteus*, *Plasmodium* and *Leucocytozoon*), yet geographic gaps in our regional knowledge remain. Here, we report the first haemosporidian screening of the breeding birds from Sardinia (the second-largest Mediterranean Island and a biodiversity hotspot), and the first for the insular Mediterranean in general. We examined the occurrence of haemosporidians by amplifying their mtDNA *cytb* gene in 217 breeding birds, belonging to 32 species. The total prevalence of infected birds was 55.3%, and of the 116 haplotypes recovered, 84 were novel. Despite the high number of novel lineages, phylogenetic analysis did not highlight Sardinia-specific clades; instead, some Sardinian lineages were more closely related to lineages previously recovered from continental Europe. Host-parasite network analysis indicated a specialized host-parasite community. Binomial generalized linear models (GLMs), performed at the community level, suggested an elevational effect on haemosporidian occurrence probability (negative for *Haemoproteus*; positive for *Leucocytozoon*) likely due to differences in the abundance of insect vectors at different elevations. Furthermore, a GLM revealed that sedentary birds showed a higher probability of being infected by novel haplotypes and long-distance migrants showed a lower probability of novel haplotype infection. We hypothesize that the high diversity of haemosporidians is linked to the isolation of breeding bird populations on Sardinia. This study adds to the growing knowledge on haemosporidians lineage diversity and distribution in insular environments and presents new insights on potential host-parasite associations.

Keywords: avian malaria; genetic diversity; haemosporidian; host-parasite association; MalAvi; mediterranean; sardinia

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1. Introduction

Blood parasite pathogenic potential can negatively affect wild bird's survival probability and consequently can also affect population dynamics [1–4]. By extension, parasite infections can have conservation impacts, particularly for small populations or declining bird species [3]. Avian blood parasites (Apicomplexa: order Haemosporida) represent one of most studied vector-borne parasite systems in birds and studies of avian haemosporidians have noticeably increased since the 2000's. This increase is in no small part due to the development of molecular techniques for haemosporidian detection, such as specific PCR protocols, which usually concern the three genera *Plasmodium*, *Haemoproteus* and *Leu-*

cocytozoon [5]. The insect vectors of these haemosporidian genera belong to different dipteran families: mosquitos (Culicidae) are known to be *Plasmodium* vectors, both louse flies (Hippoboscidae) and biting midges (Ceratopogonidae) are *Haemoproteus* vectors, and black flies (Simuliidae) are vectors for *Leucocytozoon* [6,7]. These insect vectors have a near global distribution and have different ecological requirements (e.g., related to variations in water availability or temperature). In addition, it appears that some haemosporidian lineages are either generalists (found in many avian host species) or specialists (found in very few, or single host species) [8]. These factors collectively mean that different geographic regions can have very different parasite communities infecting avian hosts. Indeed, the remarkable genetic richness in haemosporidian lineages (i.e., $n = 4169$ MalAvi haplotypes, as of November 2020 [5]) has provided an ideal framework to assess host-parasite dynamics. For example, genetic diversity reaches higher values in areas with high environmental heterogeneity and rich avian host communities [9], suggesting that there is still much diversity to discover and describe in such environments. An extensive literature which comprises both single-species [9,10] and multi-species/communities' studies [11,12] also points to a very complicated framework affected by intertwined ecological factors, to include host-parasite relationships [13,14], host ecological or ethological traits [15], insect vector abundance [16] and climate influence [17]. Moreover, the framework is further complicated by unique dynamics, inherent to the biogeographic region in which studies are conducted, such as isolation-colonization events [18,19]. Thus, studies from new regions may be useful in disentangling the determining factors influencing haemosporidian infections and allowing assessments of whether those factors are generalized (e.g., similar across regions) or unique [18].

The Western Palearctic, from Iberia and Morocco in the west to the Caucasus and Ural Mountain regions in the east, is among the most investigated biogeographic regions for genetic diversity of avian haemosporidians [18,20–24]. However, there are still numerous gaps within this extensive geographic range. This includes very poorly sampled areas such as the Italian Peninsula, where molecular diversity data for haemosporidians are limited to a few host species [11,25,26], and areas completely overlooked as is the case for insular land masses in the Mediterranean Sea. Indeed, to our knowledge, no bird community from the insular Mediterranean has ever been investigated for avian haemosporidians via molecular methods, although a few studies focused on single host species [27] or migrating birds [28] have been conducted on very small islands or islets.

One such overlooked island is Sardinia, the second-largest (after Sicily) in the Mediterranean Sea. This island is ecologically diverse, ranging from coastal habitats to a mountainous interior, which results in varying microclimates. During the Pleistocene, the Western Mediterranean islands (i.e., Balearic Islands, Corsica, Tuscan Archipelago and Sardinia) acted as a glacial refugium for several organisms [29], and through subsequent isolation mechanisms became an important biodiversity hotspot in Europe [30]. Sardinia hosts a very rich avifauna including 163 breeding bird species [31], of which several are endemic taxa or have unique genotypes relative to mainland populations [32–36]. In addition, the breeding avifauna is comprised of both sedentary species (or populations) and migratory species.

In this study we investigated, for the first time, the avian malaria parasites of Sardinian breeding bird communities through the use of molecular tools, in order to: (i) describe the prevalence and the local genetic diversity of parasites; (ii) assess the uniqueness of Sardinian lineages by comparison to described lineages and their distribution using MalAvi [5]; and (iii) analyze the host-parasite relationships that occur in this area and test the influence of possible environmental or host-species traits that may affect infection occurrence (i.e., individual infection status). Given its insular nature and largely endemic biodiversity, we hypothesize that the Sardinian breeding bird community will harbor a high diversity of avian haemosporidian lineages. We further expect this haemosporidian community will include novel haplotypes, but also expect the occurrence of widespread generalist parasite lineages from the mainland.

2. Methods

2.1. Study Area and Fieldwork

Sardinia is the second-largest Mediterranean island (23,821 km²), positioned in the western part of the basin. This insular land mass is about 180 km from continental land masses (both Europe and Africa). Blood samples were collected from wild birds (217 individuals) in seven localities (Figure 1). Sample sites were selected to be representative of the island's elevational gradient (ranging from 3 m asl to 1309 m asl) and different environments to include reed-dominated wetland, woodland (e.g., Mediterranean sclerophyllous and Tyrrhenian cork-oak forests), agricultural mosaics and montane shrublands. Fieldwork took place during the breeding season in 2018, in the last two weeks of May. Birds were captured using mist nets and were ringed in order to avoid double sampling, under permits of the Sardinian Assessorato della Difesa dell'Ambiente (Prot. 822/18 of 15 Jan 2018, Det. 10616/288 of 11 May 2018) and of the Italian Istituto Superiore per la Protezione e la Ricerca Ambientale (Prot. 9447 of 26 Apr 2018). Approximately 50–100 µL of whole blood was drawn from the brachial vein by puncturing it with a sterile 25-gauge needle and preserved in Queen's Lysis buffer [37]. Sampled birds represented 20% of Sardinian breeding species [31]. As the Sardinian breeding bird community is composed of species with very different migratory patterns (sedentary, short-distance (intra-Palaearctic), or long-distance (trans-Saharan), we collected samples only from those individuals showing morphological evidence of breeding status (i.e., cloacal protuberance or brood patch), thus avoiding the inclusion of birds that were actively migrating in the analysis. Nomenclature used is in accordance with IOC World Bird List [38]. All voucher specimens (i.e., collected blood, See Table S1) collected are deposited in the Lab of Zoology at the University of Piemonte Orientale, Vercelli, Italy.

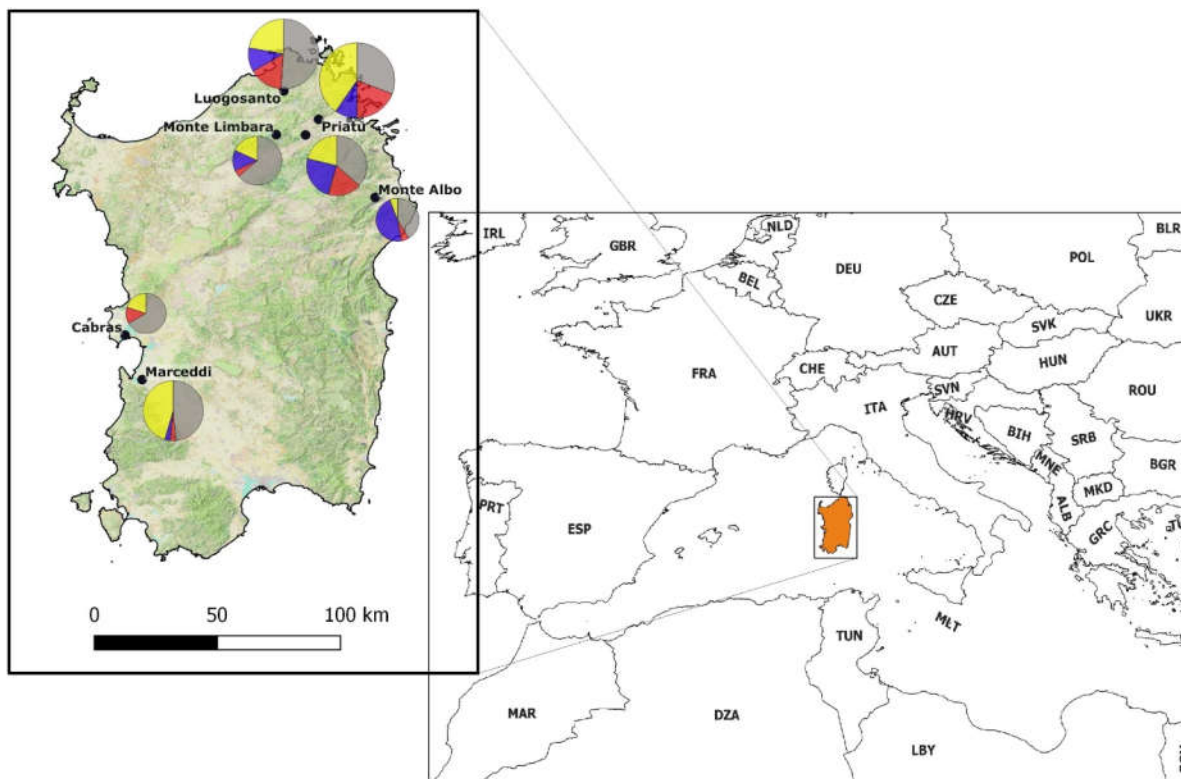


Figure 1. Study area location in the Mediterranean basin. Sampling localities (black dots) are Cabras (3 m asl), Luogosanto (216 m asl), Marceddi (3 m asl), Monte Albo (780 m asl), Monte Limbara (1309 m asl), Priatu (308 m slm), Telti (368 m asl). Pie diagrams show the proportion for each site of uninfected birds (grey) and birds infected by *Haemoproteus* (yellow), *Leucocytozoon* (blue) and *Plasmodium* (red).

2.2. Molecular Analyses

DNA was extracted using the E.Z.N.A. tissue extraction kit (Omega Bio-Tek, Norcross, GA, USA) following the standard protocols, with a final elution of 75 μ L to increase overall DNA yield. Polymerase chain reaction (PCR) was used to identify haemosporidian infection by amplifying a fragment of the mtDNA cytochrome b (*cytb*) gene, and multiple primer pairs [8] were used to amplify across known avian haemosporidian genetic diversity following molecular protocols previously described in Harvey and Voelker [17]. A single forward primer, UNIVF, was combined with each of three reverse primers UNIVR1, UNIVR2, UNIVR3, to amplify all the three genera of avian haemosporidians: *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. To verify an infection, 4 μ L of the final PCR product was run on 1.8% agarose gel. All reactions were performed with negative (double-distilled H₂O) and positive controls (infected individuals determined after three repeated PCR) to evaluate the validity of the PCR and detect possible contamination. PCR fragments were sequenced in both directions by MacroGen Europe labs (Amsterdam, The Netherlands).

2.3. Phylogenetic and Statistical Analysis

The sequences of 479 base pairs of the *cytb* gene obtained were controlled through the visualization of electropherograms using FinchTV (Geospiza Inc., Seattle, WA, USA <http://www.geospiza.com> (accessed on January 2020)). Any sequences containing ambiguities or evident sequencing errors (e.g., insertions, deletions, and faulty end-parts) were discarded and the samples were reprocessed with a new PCR and sequencing. We utilized MEGA X [39,40] to align and trim the sequences, and DnaSP v5.1 [41] to identify unique haplotypes and to reconstruct single infection haplotypes [42]. Double infections were identified by the presence of multiple peaks in chromatograms, these sequences were phased using the Phase 2.1 [43] implementation in DnaSP 5.10.1 following the procedure described by Harvey and Voelker [17]. The reconstructed single infection haplotypes were subjected to BLAST analysis on the MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/blast.html> (accessed on May 2020)) and any sequences that had a match <97% were excluded from the data set.

The obtained haplotypes were blasted in GenBank and MalAvi [5] databases using the NCBI BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> (accessed on May 2020)), and the sequences with one or more nucleotide substitutions were identified as new lineages according to the MalAvi Public Database. All comparisons to MalAvi are based on records accessed in November 2020 (Version 2.4.7, 6 October 2020). All sequences were deposited in both the MalAvi database (<http://mbio-serv4.mbioekol.lu.se/avianmalaria> (accessed on May 2020)) and GenBank (Table S1).

Genetic distances (*p-distance*) within and between haemosporidian genera were calculated, and phylogenetic relationships were reconstructed in MEGA X. Phylogenetic trees were calculated by Neighbor-Joining with 1000 bootstrap replicates (pairwise deletion), and by Bayesian Inference utilizing the Markov Chain Monte Carlo (MCMC) method implemented in MrBayes 3.1 [44], and implementing the best model estimate of sequence evolution (GTR+I+G) acquired from jModeltest v2.1 [45]. Our MrBayes analysis was run for 4,000,000 generations with a burn-in of 25%. A *Hepatocystis* sp. (GenBank KC262858) was used as an outgroup. Phylogenetic trees were edited in FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/> (accessed on May 2020)).

To highlight the host-parasite degree of specialization we calculated the network level specificity index (H_2') for bird-haemosporidian communities and the species-level specificity index (d') for each parasite lineage using the *bipartite* package [46]. These indices range from 0 to 1, indicating a generalized network for H_2' values close to 0, and a specialized network for H_2' close to 1, and no specialization ($d' = 0$) and high specialization ($d' = 1$) at the parasite lineage level. We compared observed (calculated on our data) with

expected H_2' and d' (estimated for random interactions) after generating 10,000 null models of the network, using the t-test in *bipartite*.

In order to evaluate the relationships between haemosporidian occurrence and environmental or species features within the Sardinian bird community, we ran binomial Generalized Linear Models (GLM) using the logit link function as implemented in R 3.6.1 [47]. Firstly, we assessed sampling site elevation as an environmental explanatory variable, in affecting the occurrence probability for each haemosporidian genus (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) as response variables (i.e., individual infection status; $n = 217$). Secondly, we tested species migratory patterns (sedentary, short-distance migrant or long-distance migrant), as an explanatory variable in predicting the occurrence probability of Sardinian-only haplotypes (i.e., those lineages that were found for the first time in this study). We considered novel Sardinian haplotype occurrence as a trait that points to haemosporidian geographic uniqueness. In this analysis, we included only infected individuals ($n = 119$), as haemosporidian-free individuals are not informative in terms of environmental or geographic features that may drive haplotype distribution. Among all performed models, no data overdispersion was detected. Models plots were made using the *visreg* R package [48].

3. Results

3.1. Prevalences of Haemosporidian Parasites

In total, we analyzed 217 individuals representing 32 bird species (three non-Passeriformes and 29 Passeriformes) belonging to 19 avian families. All of the individuals of the non-Passeriformes species were negative for infection (*Caprimulgus europaeus*, $n = 1$; *Dendrocopos major*, $n = 4$; *Falco tinnunculus*, $n = 1$), as were all individuals of six species of Passeriformes (*Carduelis carduelis*, $n = 1$; *Hirundo rustica*, $n = 1$; *Lullula arborea*, $n = 1$; *Phylloscopus collybita*, $n = 1$; *Regulus ignicapilla*, $n = 2$; *Curruca sarda*, $n = 3$).

Across all other species we found 119 positive individuals (total prevalence of 55.3%). Rates of infection varied from 14.3% (*Emberiza calandra*, $n = 7$ total samples) to 100% (*Lanius senator*, $n = 2$; *Muscicapa tyrrenica*, $n = 1$; *Garrulus glandarius*, $n = 7$; *Turdus viscivorus*, $n = 1$) (Table 1). The prevalence in Passeriformes families for which we analyzed more than five individuals significantly varies from 38.5% in Emberizidae to 100% in Corvidae (Pearson's Chi²-test: $\chi^2 = 50.953$, $df = 9$, $p \leq 0.001$).

Table 1. List of avian families and species tested for infection by haemosporidians, along with migratory patterns (S = Sedentary; SD = Short-distance migrant; LD = Long-distance migrant); number of sampled individuals (n); haemosporidian prevalence (prev.); number of positive (+) and negative (−) individuals for haemosporidian infection; number of infection relative to *Haemoproteus* (H), *Leucocytozoon* (L) *Plasmodium* (P) haplotypes; and list of haemosporidian haplotypes detected. SA precedes novel Sardinian haplotypes, followed by a unique number, and a designation of which parasite lineage is represented (e.g., SA14_H is the 14th novel lineage we identified, representing *Haemoproteus*). Haplotypes in bold highlight new host-parasite associations for known MalAvi lineages.

Species	Migratory Pattern	n	prev.	Infections			Haplotypes Detected	
				+	−	P		
Caprimulgidae		1	0%	−	1	−	−	
<i>Caprimulgus europaeus</i>	LD	1	0%	−	1	−	−	
Picidae		4	0%	−	4	−	−	
<i>Dendrocopos major</i>	S	4	0%	−	4	−	−	
Falconidae		1	0%	−	1	−	−	
<i>Falco tinnunculus</i>	S	1	0%	−	1	−	−	
Lanidae		2	100%	2	−	2	−	
<i>Lanius senator</i>	LD	2	100%	2	−	2	−	RBS3_H
Corvidae		7	100%	7	−	7	−	
<i>Garrulus glandarius</i>	S	7	100%	7	−	7	−	CIRCUM05_H, GAGLA05_H, SA14_H, SA32_H, SA33_H, SA90_H, SA91_H
Paridae		29	55.2%	16	13	3	10	9
<i>Periparus ater</i>	S	4	50%	2	2	−	2	1
								PARUS20_L , SA88_L, SA89_L, SGS1_P

<i>Cyanistes caeruleus</i>	S	13	38.5%	5	8	2	5	–	PARUS1_H, PARUS14_L, PARUS4_L, SA18_L, SA44_L, SA45_L, SA53_L, SA54_H
<i>Parus major</i>	S	12	75%	9	3	1	3	8	PARUS1_H, PARUS19_L, SA07_P, SA24_P, SA36_L, SA39_P, SA40_L, SA48_L, SA49_L, SA50_L, SA70_P, SA71_P, SA73_L, SA74_L, SGS1_P
Alaudidae		1	0%	–	1	–	–	0	
<i>Lullula arborea</i>	SD	1	0%	–	1	–	–	0	
Hirundinidae		1	0%	–	1	–	–	0	
<i>Hirundo rustica</i>	LD	1	0%	–	1	–	–	0	
Cettiidae		4	25%	1	3	–	–	1	
<i>Cettia cetti</i>	S	4	25%	1	3	–	–	1	SGS1_P
Phylloscopidae		1	0%	–	1	–	–	0	
<i>Phylloscopus collybita</i>	SD	1	0%	–	1	–	–	0	
Acrocephalidae		22	63.6%	14	8	14	–	–	
<i>Acrocephalus scirpaceus</i>	LD	22	63.6%	14	8	14	–	–	BRW1_H, ARW1_H, MW1_H, SA10_H, SA20_H, SA21_H, SA72_H, SA76_H, SA77_H, SA78_H
Sylviidae		39	59.0%	23	16	16	5	3	
<i>Sylvia atricapilla</i>	SD	15	86.6%	13	2	11	2	–	SA13_H, SA15_H, SA17_H, SA22_H, SA25_H, SA26_H, SA27_H, SA42_H, SA43_H, SA52_H, SA79_L, SA80_L, SA86_L, SA87_L, SYAT01_H, SYAT02_H, SYAT22_L, SYAT44_H, SYBOR07_L
<i>Curruca melanocephala</i>	S	15	53.3%	8	7	5	1	3	SYAT22_L, SYMEL02_H, SA08_H, SA09_H, SA34_H, SA35_P, SA83_L, SGS1_P
<i>Curruca subalpina</i>	LD	6	33.3%	2	4	–	2	–	SYAT22_L, SFC8_L
<i>Curruca sarda</i>	SD	3	0%	–	3	–	–	–	
Regulidae									
<i>Regulus ignicapilla</i>	SD	2	0%	–	2	–	–	2	
Sturnidae		5	40%	2	3	–	–	2	
<i>Sturnus unicolor</i>	S	5	40%	2	3	–	–	2	SA03_P, SYAT05_P
Turdidae		15	73.3	11	4	2	–	9	
<i>Turdus merula</i>	SD	14	71.4%	10	4	1	–	9	SA01_P, SYAT05_P, TURDUS2_H
<i>Turdus viscivorus</i>	SD	1	100%	1	–	1	–	–	CUKI1_H
Muscicapidae		22	54.5%	12	10	10	1	2	
<i>Muscicapa tyrrenica</i>	LD	1	100%	1	–	1	–	–	SFC1_H
<i>Erithacus rubecula</i>	SD	16	62.5%	10	6	9	–	2	ROBIN1_H, SA02_H, SA19_P, SA30_H, SA84_H, SGS1_P
<i>Saxicola rubicola</i>	SD	5	20%	1	4	–	1	–	SFC8_L
Passeridae		27	44.4%	12	15	2	6	7	
<i>Passer hispaniolensis</i>	S	27	44.4%	12	15	2	6	7	PARUS69_L, PADOM23_H, SA06_L, SA28_L, SA47_L, SA55_L, SA56_P, SA57_P, SA58_P, SA59_L, SA60_P, SA61_P, SA62_H, SA63_P, SA64_L, SA65_P, SGS1_P
Fringillidae		20	70.0%	14	6	6	8	–	
<i>Fringilla coelebs</i>	SD	6	83.3%	5	1	4	1	–	BRAM2_L, CCF6_H, SA16_H, SA29_H, SA31_H, SA46_H
<i>Chloris chloris</i>	SD	3	66.7%	2	1	1	1	–	CARCHL01_H, CARCHL04_L
<i>Linaria cannabina</i>	SD	6	83.3%	5	1	–	5	–	CARCHL04_L, SA04_L, SA06_L, SA66_L, SA67_L, SA68_L, SA69_L, SA92_L
<i>Carduelis carduelis</i>	SD	1	0%	–	1	–	–	–	
<i>Carduelis corsicana</i>	S	4	50%	2	2	1	1	–	CARCHL04_L, SA11_H,
Emberizidae		13	38.5%	5	8	2	2	2	
<i>Emberiza calandra</i>	S	7	14.3%	1	6	–	–	1	SA05_P
<i>Emberiza cirius</i>	S	6	66.7%	4	2	2	2	1	AEDVEX01_P, PARUS69_L, EMCIR01_H,, SA12_H, SA23_L

The number of haemosporidian lineages detected in each host species ranged from one (several species), to 19 in *Sylvia atricapilla*; other species with a high number of lineages are *Parus major* (15) and *Passer hispaniolensis* (14).

In total, we detected 116 unique haplotypes, 84 (72.4%) of which were novel, as they had at least a one base-pair difference from the lineages represented in the MalAvi database, while 32 (27.6%) haplotypes have previously been deposited in MalAvi (Table S1). The identified lineages were attributed to the genera of *Haemoproteus* ($n = 55$: 36 novel; 19 on MalAvi), *Plasmodium* ($n = 20$: 17 novel; 3 on MalAvi), and *Leucocytozoon* ($n = 41$: 31 novel; 10 on MalAvi).

Co-infections were recovered for 13 individuals of four species. *Haemoproteus* and *Plasmodium* co-infections were recovered from two individuals (*Currucula melanocephala* and *Erithacus rubecula*). *Leucocytozoon* and *Plasmodium* were recovered simultaneously in eight individuals (one *Emberiza cirius*, three in both *Parus major* and *Passer hispaniolensis* and one *Periparus ater*). *Haemoproteus* and *Leucocytozoon* were recovered in three individuals (two *Cyanistes caeruleus* and one *Muscicapa tyrrenica*) (Table S1).

Prevalence of Haemosporidian infections in the sampled localities (Table 2; Figure 1) varied from 33.3% (Cabras) to 69.2% (Priatu), and in two localities (Cabras and Marceddì, both about 20 km from the coast and effectively at sea level), we did not find *Leucocytozoon* infections. *Haemoproteus* infections were the most common in all sampling localities except in Monte Albo (780 m asl), where we identified an evident dominance of *Leucocytozoon* infections (Pearson's Chi²-test: $\chi^2 = 9.8$, $df = 2$, $p = 0.0074$) of 80% versus 10% each for *Plasmodium* and *Haemoproteus*.

Table 2. Sampling localities with information about elevation, environmental features, number of sampled host individuals (n); overall haemosporidian prevalence (prev.); haplotypes number found for *Haemoproteus* (H), *Leucocytozoon* (L) and *Plasmodium* (P).

Locality	Elevation m a.s.l.	Environment	n	prev.	H	L	P
Cabras	3 m	Agricultural mosaic	15	33.3%	4	-	5
Marceddì	3 m	Reed-dominated Wetland	33	51.5%	23	2	2
Luogosanto	216 m	Woodland	45	48.9%	18	9	7
Priatu	308 m	Woodland	52	69.2%	30	9	13
Telti	370 m	Agricultural mosaic	32	65.6%	7	4	6
Monte Albo	780 m	Montane shrublands	17	58.8%	1	8	1
Monte Limbara	1309 m	Montane shrublands	22	36.4%	4	7	1

Some host species showed a high number of infection occurrences: *Sylvia atricapilla* with 23 (17 *Haemoproteus* and six *Leucocytozoon*); *Acrocephalus scirpaceus* with 22 (*Haemoproteus*); *Passer hispaniolensis* with 21 and *Parus major* with 20 (Table S1).

Among the haemosporidian lineages detected in this study and recorded in MalAvi, some are relatable to morphologically described species. *Plasmodium* lineages SGS1 (*Plasmodium relictum*) and SYAT05 (*Plasmodium vaughani*) were the most frequently found: 10 times each in six species (from five families) and two species (from two families) respectively (Table 1). Another frequently detected haplotype, ARW1 (*Haemoproteus belopolskyi*), was found nine times but only in *Acrocephalus scirpaceus*. We also detected another seven lineages attributable to a described parasite species: MW1 that still pertains to *Haemoproteus belopolskyi*; PARUS1 to *Haemoproteus majoris*; ROBIN1 to *Haemoproteus attenuates*; SFC1 to *Haemoproteus balmorali*, SYAT01 and SYAT02 to *Haemoproteus parabelopolskyi* and TURDUS2 to *Haemoproteus minutus*.

We also identified 13 new host-parasite associations compared to data published on MalAvi (Table 1).

The host-parasite network is reported in Figure 2 (and see Figure S1). The host-parasite network-level specificity index in Sardinia showed a high value ($H_2' = 0.756$), significantly higher than the null model value (0.141; $p \leq 0.001$). The species-level specificity index (d') calculated on our dataset indicated a high specialization (≥ 0.70 , null model value = 0.455; $p < 0.001$) for 12 haemosporidian lineages (Supporting information Table S2), three which are new lineages from Sardinia and nine having been previously described. Of the nine previously described, five were associated with a single species: CIRCUM05 found exclusively in *Garrulus glandarius* (five occurrences), RBS3 in *Lanius senator* (two occurrences), ROBIN1 in *Erithacus rubecula* (seven occurrences), ARW1 in *Acrocephalus scirpaceus* (nine occurrences), CUKII in *Turdus viscivorus* (two occurrences) (Table S1).

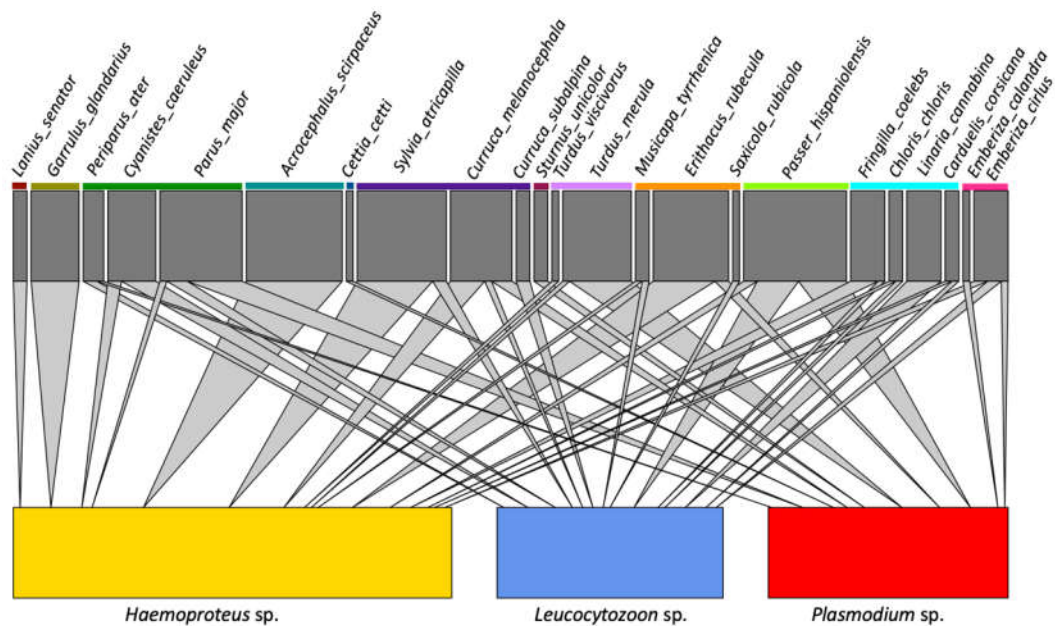


Figure 2. Host-parasite networks of avian and haemosporidian Sardinian communities; parasites were grouped by genus. Interactions between a given host species and a haemosporidian genus is denoted with a cone; the size of the cone is proportional to the number of infections detected in each species. Colored lines under host species names indicate taxonomic family groupings.

3.2. Genetic Diversity and Phylogeny of Haemosporidian Lineages

p-distances calculated for each of the three haemosporidian genera were 0.054 (± 0.006) in *Haemoproteus*, 0.062 (± 0.006) in *Leucocytozoon* and 0.070 (± 0.007) in *Plasmodium*. Between group mean distance across genera was 0.114 (± 0.010) for *Haemoproteus* and *Plasmodium*, 0.160 (± 0.014) between *Haemoproteus* and *Leucocytozoon*, and 0.166 (± 0.014) between *Plasmodium* and *Leucocytozoon*.

The Neighbor-joining tree (Figure 3) and Bayesian trees (GTR+G+I model) showed a similar topology. The phylogenetic reconstruction of avian haemosporidian parasite lineages clustered with strong support in three major clades representing the three genera: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. Notably, most of the novel Sardinian lineages do not form monophyletic clusters; instead, many are more closely related to more widespread lineages than they are to other Sardinian-only lineages.

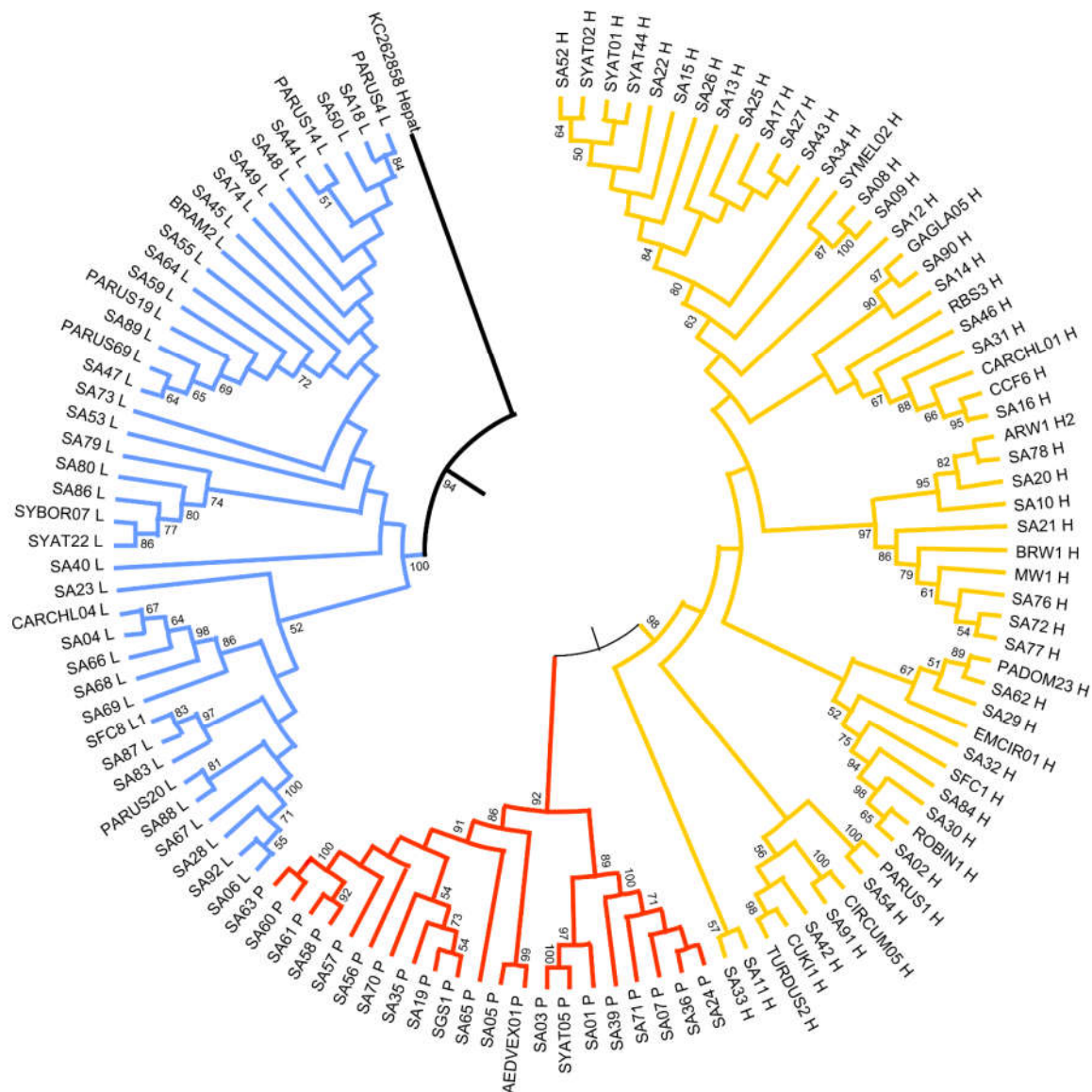


Figure 3. Neighbor-joining tree of the partial *cytb* gene (470 bp) of *Plasmodium* (red), *Haemoproteus* (yellow), and *Leucocytozoon* (blue) sequenced from Sardinian birds. Bootstrap values ($\geq 50\%$) are provided. “SA” precedes novel lineages found in this study; other lineages are named based on their match to MalAvi lineages.

3.3. Haemosporidian Occurrence Predictors

Binomial generalized linear models (GLMs) performed to evaluate haemosporidian genus occurrence probability in the Sardinian bird community showed a significant influence of sampling site elevation. This relationship was negative for *Haemoproteus* (decreasing occurrence with increasing elevation) and positive for *Leucocytozoon*; no effect of elevation was evident for *Plasmodium* (Table 3, Figure 4). A model assessing novel Sardinian haplotypes occurrence probability showed an effect related to host species migratory patterns, where the three migratory categories (sedentary, short-distance migrants and long-distance migrants) respectively display higher, intermediate and lower infection probabilities (Table 3, Figure 5).

Table 3. Binomial generalized linear models (GLMs) results. The first three (each parasite genus) assess the effects of sample site elevation on haemosporidian occurrence probability, across the entire Sardinian bird community that we sampled. The last result shows the influence of species migratory pattern in affecting novel Sardinian haplotype occurrence probability in infected individuals.

<i>Haemoproteus</i> Occurrence	$\beta \pm ES$	Z	p
intercept	-0.523 ± 0.211	-2.477	0.013
Sample Site Elevation	-0.001 ± 0.0005	-2.141	0.032
<i>Leucocytozoon</i> Occurrence	$\beta \pm ES$	Z	p
intercept	-2.231 ± 0.288	-7.741	<0.001
Sample Site Elevation	0.001 ± 0.0004	2.713	0.007
<i>Plasmodium</i> Occurrence	$\beta \pm ES$	Z	p
intercept	-1.468 ± 0.258	-5.689	<0.001
Sample Site Elevation	-0.001 ± 0.001	-0.940	0.347
Novel Haplotypes Occurrence	$\beta \pm ES$	Z	p
Long-distance	-1.674 ± 0.629	-2.661	0.008
Short-distance	1.460 ± 0.694	2.104	0.035
Sedentary	2.175 ± 0.690	3.152	0.002

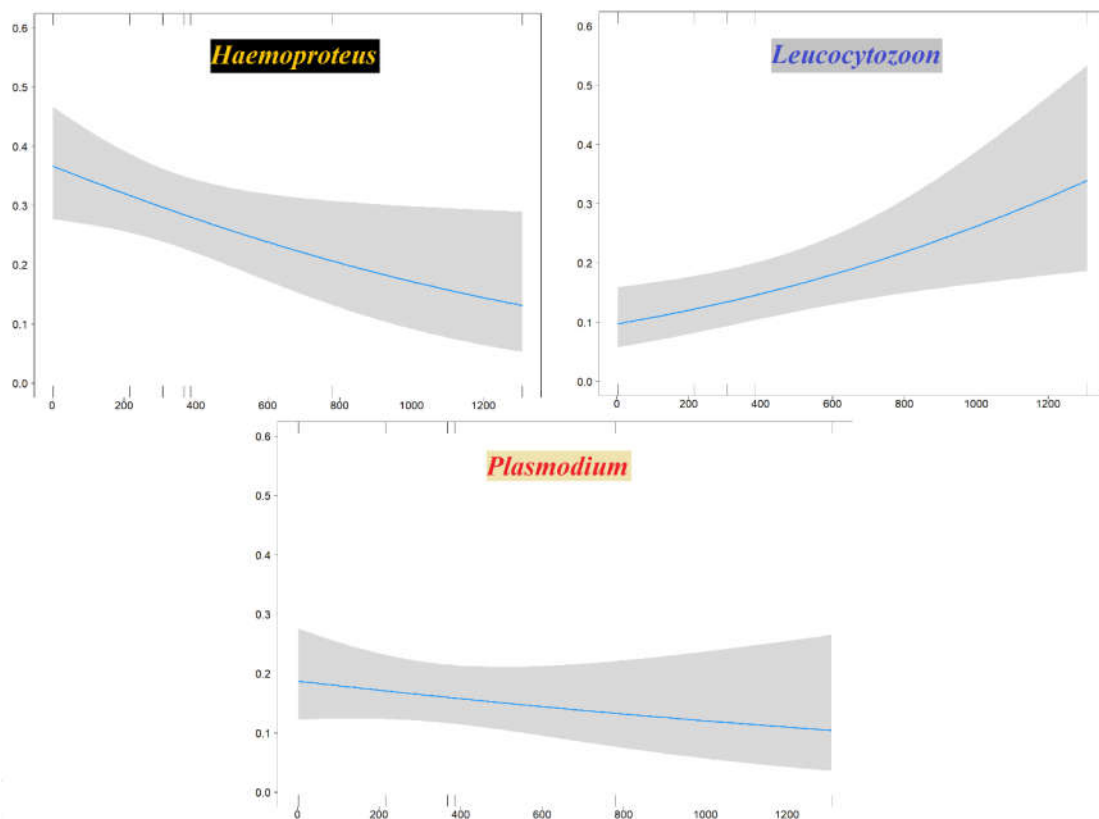


Figure 4. Plots showing the effects of the sampling site elevation (X axis: meters asl) on the occurrence probability (Y axis) of *Haemoproteus* (left), *Leucocytozoon* (right), *Plasmodium* (bottom) on Sardinian birds.

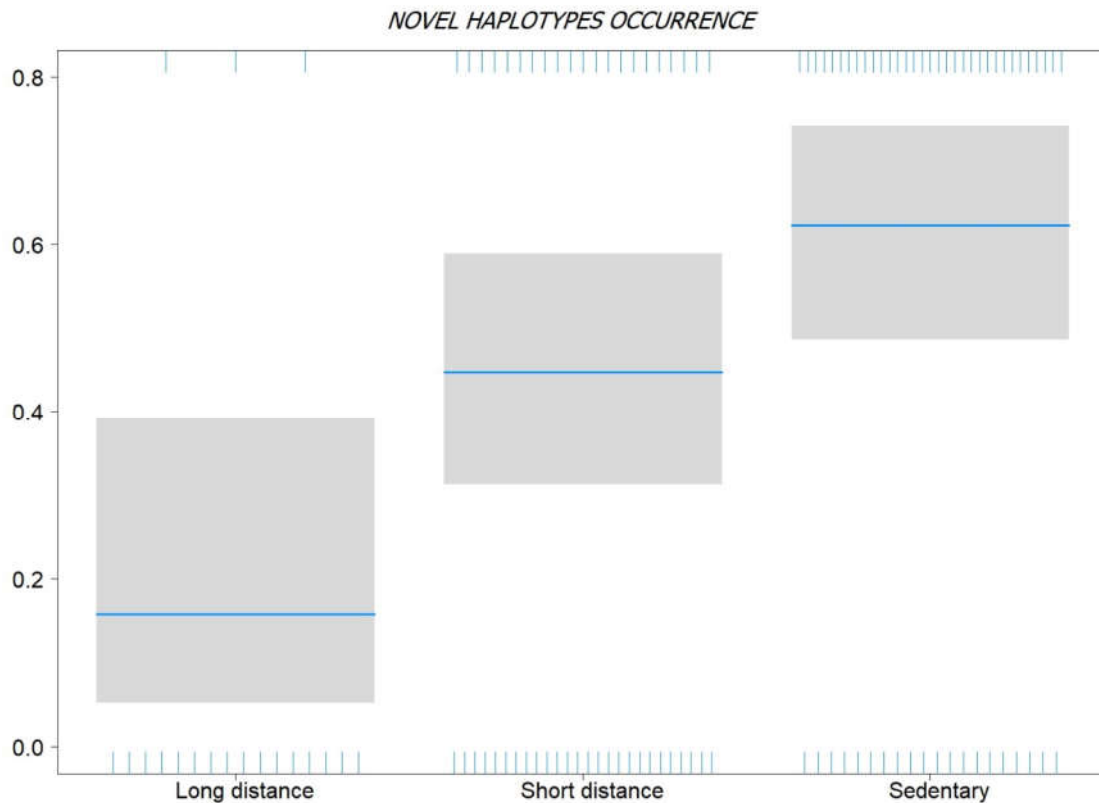


Figure 5. Plot showing the effects of species migratory patterns (X axis) on the occurrence probability of novel haplotypes found in Sardinia (Y axis) in those birds infected by haemosporidians.

4. Discussion

4.1. Sardinian Haemosporidian Diversity

The present study provides the first molecular screening of avian malaria parasites in the bird breeding community from Sardinia, and in general the first from Mediterranean islands.

Our analyses detected haemosporidian infections in 119 individuals from 23 species (of 32 bird species analyzed), and recovered 116 haplotypes, 84 of which are new to MalAvi. In nine host species, we did not observe any infections, and, although the number of birds sampled in some species was low, the negative results from *Caprimulgus europaeus*, *Dendrocopos major*, *Lullula arborea*, *Regulus ignicapilla*, and *Curruca sarda* is consistent with there being no molecular evidence of infections recorded to date (see MalAvi). We identified for the first time a haemosporidian parasite infection in four species: *Muscicapa tyrrhenica*, *Saxicola rubicola*, *Carduelis corsicana* and *Lanius senator*. For the latter species, a previous association was indirectly related to louse flies [49].

In most of the examined host species, we detected prevalence levels comparable to those previously published, such as high prevalence of infections of *Plasmodium* in *Turdus merula* [50] and of *Haemoproteus* in *Garrulus glandarius* [51].

In other species, we detected an intermediate level of prevalence, which is comparable to other studies. For example, the prevalence level of 63.6% in *Acrocephalus scirpaceus*, a species typical of marshes dominated by common reed (*Phragmites australis*) and sampled only in Marceddì (reed-dominated wetlands), is somewhat lower than that 84.6% detected in Spain by Fernandez et al. [52], but it is higher than the 33.1% detected in Portuguese wetlands by Ventim et al. [53]. The most highly sampled host species in our study was *Passer hispaniolensis*, which showed a prevalence of 44.4%. This level of prevalence is

generally similar to the value (55.6%) reported for Turkish populations of this species [54]. However, interestingly, the infection rates between that study and ours differed with respect to levels of parasite genus infections: our *Passer hispaniolensis* were infected by, in ascending order, *Haemoproteus* (13.3%), *Leucocytozoon* (40%) and *Plasmodium* (46.7%), while in Turkey *Plasmodium* infections were lacking and *Haemoproteus* was the main infecting genus.

We found substantial differences in infection rate across bird families (Table 1) suggesting that certain families experience a higher amount of parasite infections than others do. This result is in line with findings reported by for example, Harvey and Voelker [17] in Benin, Clark et al. [18] in the Western Palearctic, and Bodawatta et al. [19] in both New Guinea and the Canary Islands. Despite these differences being observed across diverse regions, the factors that may affect the haemosporidian prevalence at the host family level have not yet been identified [20].

The host-parasite network in Sardinia indicated a high value of the specificity H_z' index (0.756), which suggests that the Sardinian host-parasite community is specialized, and this result differs from the more generalized host-parasite community observed in the Canary and New Guinea Islands [19]. These different community structures could be due to the differences in bird species and environmental diversity: i.e., the Canary Islands are smaller and contain fewer breeding species than Sardinia, while the more diverse bird community in New Guinea was sampled over a long elevational gradient (from 200 to 3700 m asl).

When considering the specialization of individual haemosporidian lineages, we found some lineages that showed a high d' species-level specificity index [46]. In particular, a high value ($d' \geq 0.70$) was found for three new Sardinian lineages (SA11_H, SA03_P, SA05_P). Another eight cases of specialization pertain to previously described lineages showing a high specificity, that was confirmed in two cases (CUKI1 and SYATT44), while high specificity was not a general character in five lineages that were also found in other host species sampled elsewhere ([50]; MalAvi database). These values could indicate an artifact due to sampling or a trend to specialization in an insular context. Overall, the number of sampled Sardinian birds per species in some cases are low and there is evidence to suggest that wider sampling, on both islands and in mainland areas, could increase the chance of detecting these apparently novel or rare haplotypes [55].

4.2. Lineage Distributions

While we detected 84 haemosporidian lineages from Sardinia that are novel to MalAvi, we also detected 32 lineages previously described in other studies. Of these 32, only four were shared with the Italian peninsula and only two with Tunisia; these are the closest mainland countries to Sardinia. The most observed haplotypes in our samples are SGS1 and SYAT05. The former is also the most widespread haplotype in the world, is ascribable to *Plasmodium relictum*, and has been reported in 127 different host species, encompassing 73 genera and 32 families (MalAvi). SYAT05 (*Plasmodium vaughani*), that we detected in two species (with 10 occurrences), is another common lineage reported from 19 different countries (including Italy) for 33 host species, encompassing 23 genera and 16 families (MalAvi). We identified the presence of *Leucocytozoon* sp. SYAT22 in three different bird species on Sardinia; this lineage has been detected from the Canary Islands to Sweden [56–58].

Our results also provide new insights regarding specific host-parasite relationships. For example, *Haemoproteus* haplotypes CIRCUM05 and GAGLA05 were found only in *Garrulus glandarius* (a corvid species) in this study, which is consistent with them having only been recorded from Corvidae species to date (MalAvi). This may indicate a close relationship between these haplotypes and Corvidae, indicative of some level of host-parasite specificity, and by extension parasite specialization. Similar host-specificity appears evident for SYAT44, which has been only recorded from *Sylvia atricapilla*, both in Sardinia and Europe, for RBS3 which appears to parasitize only *Lanius* host species [59,60], for

SYLMEL02 [52] and SYBOR07 [51,57,60–62] which have only been found in *Sylvia* hosts, for PADOM23 found only in *Passer* hosts [51,53,63], for AEDVEX1 found only in *Emberiza* hosts [24] and for BRAM2 found only in *Fringilla* hosts [51,57,60]. Conversely, some haemosporidian lineages are less specific, and include SFC1 (*Haemoproteus balmorali*), which we only detected in *Muscicapa tyrrenhennica* (a Muscicapidae), but which has also been observed in Turdidae and Paridae (MalAvi).

4.3. Genetic Diversity

While phylogenetic reconstruction readily identified the three parasite genera and their relationships to one another, the relationship of haplotypes in each genus is generally unresolved. Therefore, while *cytb* is a good molecular barcode for Haemosporidian identification and characterization, it is unable to reliably disentangle phylogenetic structure within genera. This is likely attributable to both the short portion of the gene that is used as the barcode standard (479 base pairs), and the high substitution rate possible at third codon positions [64].

Haemosporidian genetic diversity observed in Sardinia is high as shown by the large number of new (84) and previously described (32) lineages detected. In this regard, the comparatively high numbers of new lineages have only been found in a few other studies, e.g., one from Benin in western Africa [17], with 85 new lineages and an infection rate of 50.9%, and another from northwestern Iberia and northwestern Africa with 111 new lineages and 56.5% of infection rate [52].

The high genetic diversity shown in the present study could reflect the complexity and the considerable molecular rate variation among haemosporidians [18,65]. Moreover, it could be explained by Sardinia as a general source of biodiversity [66], with co-evolution between host species and haemosporidian lineages being driven by isolation [67]. In this context, the last connections between Sardinia and continental land masses are dated to at least 5 mya, during the Messinian salinity crisis events [68]. Another possible factor contributing to the high number of novel lineages could be that a burst of diversification may have occurred following a mosquito vector eradication program that was enacted on Sardinia from 1946–1950 [69]. During this period, 267 metric tons of DDT (dichloro-diphenyl-trichloroethane), a wide range insecticide, were spread across the island, vegetation was cut back, and 100,000 acres of swampland were drained; the presumed reduction of mosquito breeding habitat was 99.93%, and zero cases of human malaria were reported in 1950 (the disease has been considered endemic to Sardinia since 502 B.C., with significant annual human mortality [69]). While targeting human malaria mosquito vectors (*Anopheles* sp. that breed in open water, in marshes and mountain streams), DDT would obviously have impacted avian haemosporidian vectors including not only mosquitos, but also black flies [70] and likely midges as well. Thus, this decrease of vectors could also explain the high number of novel haplotypes relative to previously known haplotypes across all three genera in our study. Indeed, Imamura et al. [71] recorded a severe bottleneck in another protozoan parasite, *Leishmania donovani*, coincident with DDT spraying campaigns for malaria eradication on the Indian subcontinent in the 1960's. Shortly after this eradication, *Leishmania donovani* populations not only increased, but they underwent significant bursts of diversification that generated new lineages (strains) of the disease agent. Finally, at least some of lineages that we have called novel might simply be an artifact of incomplete sampling on nearby continents. A new study of the avian haemosporidian community on São Tomé [72] also found high numbers of novel lineages that they termed “endemics”. In justifying the naming of endemic lineages, they referred to the nearly 650 described sub-Saharan African haemosporidian lineages in MalAvi, only some of which were present in their study. By comparison, sampling in the Western Palearctic has been far more extensive in terms of both geographic coverage and avian host diversity, and as such, we are confident that a considerable number of our novel lineages will remain so as new continental areas are sampled.

4.4. Effect of Elevation and Host Species Migratory Pattern

Two of our GLMs analyses support an effect of elevation on infection occurrence probability: a positive effect for *Leucocytozoon* and a negative one for *Haemoproteus*, while no significant effect was recovered for *Plasmodium*. The *Leucocytozoon* pattern is likely due to distributional constraints affecting its insect vectors. Indeed, the occurrence of this haemosporidian genus is positively related to elevation since in its main vector species, black flies (Simuliidae), larvae often reach high-density values in streams with high flow speed and low water temperature [73]. These stream features both tend to increase with elevation, and black fly species richness is highest near mid-level elevations (e.g., 1985 m asl in the Italian Alps [74]; 1405 m asl in Malaysia, [75]). The positive relationship between *Leucocytozoon* and elevation we found on Sardinia is consistent with other studies carried out in other geographic regions [15,76]. The decreasing occurrence of *Haemoproteus* with elevation on Sardinia is a pattern consistent with several studies which also found a negative relationship between elevation and the distribution of this genus (e.g., [77]; also see Doussang et al. [78] for a converse relationship). *Haemoproteus* is vectored mainly by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae). Biting midges should be negatively affected by low temperatures and as such should decrease with increasing elevation [79], particularly above mid-level elevations [80]. Although there is a general decrease of *Plasmodium* infections at high elevation [76], in our study a negative effect of elevation on *Plasmodium* occurrence was not statistically significant.

Finally, we found a relationship between avian host species migratory patterns and the occurrence probability of haemosporidian haplotypes found exclusively on Sardinia. Infected individuals belonging to sedentary host species, for which we would assume each one is part of an endemic population, show a higher probability of carrying haplotypes (so far) unique to Sardinia, as compared to short-distance (i.e., intra-Palaeartic) and long-distance (i.e., trans-Saharan) migrants. While this relationship might be due at least in part to the DDT eradication program mentioned above, it might also be due to the fact that long-distance migratory species spend a considerable part of the year in tropical latitudes, where haemosporidian transmission is likely not constrained by climatic conditions (i.e., temperature) for both vector and protozoan reproductive cycles [6,27]. Conversely, haemosporidian infection in sedentary species and short-distance migrants (restricted by the temperate climate) occurs mostly during spring and summer seasons [81]. This also likely reduces the possibility of the latter two categories becoming infected by parasite lineages not occurring in Sardinia (e.g., during overwintering of northern bird populations in Sardinia, or long-distance migrants passing through).

In conclusion, while the Western Palearctic is one of the most studied regions with respect to avian haemosporidian parasites, our results indicate that there is much yet to learn about their distributions, associations, and means of transmission via habitat, elevation and host life-history variables. While we have discussed 84 Sardinia-novel lineages here, we fully expect that some may prove to have wider distributions as other insular (e.g., Corsica, Sicily) or under-sampled (e.g., Italy) avian host populations are sampled. Furthermore, a focus on the insect vectors of avian haemosporidians is required to better assess haemosporidian ecological determinants, such as elevation and other environmental features to include available water sources and types for insect vector breeding. Indeed, studies focused on avian haemosporidians from other Mediterranean islands would not only complete the knowledge gap in this geographic context but would also provide for robust assessments of the comparative role of islands in their diversification.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/13/2/75/s1, Figure S1: Host-parasite network of bird species and haemosporidian lineages. Table S1: Table of lineage, host, locality and GenBank accession numbers Table S2: Value of d' species-level specificity index calculated for each lineage.

Author Contributions: G.B., I.P., M.P., G.V., conceived of and designed the study. G.B., M.P., H.L.P. and G.V. collected the samples in the field. I.P. performed the molecular and data analyses. M.C. and L.I. performed statistical analyses. I.P. and L.I. prepared the manuscripts with contributions from all other authors. All authors have read and agreed to the published version of the manuscript.

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