

# First Report of Avian Haemosporidians Infection and Associated Risk Factors in Red Jungle Fowl (*Gallus gallus*) in China

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## Short Report

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# Abstract

## Background

Avian haemosporidians are critical hematocytozoon that infect both poultry and wild birds, responsible for anemia, acute tissue degeneration, and depopulation in wild birds. Poultry and wild birds have been reported as common reservoirs of hematocytozoon, but limited information was available for red jungle fowl (*Gallus gallus*) in China. The present study firstly investigated the prevalence and molecular characterization of haemosporidians in red jungle fowls.

## Methods

Blood samples were collected from 234 red jungle fowls from Jinghong City of Yunnan province, and then the samples were stained and observed by using microscopy. The genomic DNA samples were extracted from these samples. The prevalence of avian haemosporidians was determined by the nested PCR targeting the mitochondrial cytochrome b (*cytb*) gene and staining morphology observation. Associated risk factors were analyzed by chi-square ( $\chi^2$ ) test. Molecular characterization was investigated based on phylogenetic analysis of *cytb* sequence.

## Results

Overall prevalence of avian haemosporidium was 74.8% (175/234), with *Haemoproteus enucleator*, *Leucocytozoon californicus*, *Plasmodium juxtannucleare*, and the prevalence of haemosporidians in adult fowls (81.1%, 107/132) was significantly higher ( $P=0.012 < 0.05$ ,  $df=1$ ,  $\chi^2=6.32$ ) than juvenile gulls (66.7%, 68/102). Three representative novel lineages were revealed.

## Conclusions

This study combined morphological and molecular phylogenetic analyses to identify avian haemosporidium in red jungle fowls, providing new information on the morphology, molecular epidemiology and geographical distribution of haemosporidian parasites. Our results suggested high prevalence and diverse genetic variations in these haemosporidians in fowls. To the best of our knowledge, this is the first records of haemosporidians infection in red jungle fowls in China.

## Background

Avian haemosporidians is a related species of *Plasmodium hominis* and a branch differentiated from a common ancestor earlier [1]. It has been a model group to study the mechanism of disease transmission and interspecific co-evolution for decades [2]. Avian haemosporidian parasites of the genera

*Plasmodium*, *Haemoproteus* and *Leucocytozoon* are diverse groups of vector-transmitted blood parasites that are abundant in most avian families and can cause disease [2–4]. At present, there are more than 4000 lineages defined based on the bar code sequence of mitochondrial cytochrome b gene (*cyt b*). Approximately, 2000 bird species can be infected by haemosporidians and this parasite has been found in all regions of the world except Antarctica, posing a serious threat to the health and even survival of infected poultry and birds [5].

Since the domestication of chickens, chickens have been respected by different cultures all over the world. Comparing with sheep, cattle, pigs and other livestock, chicken is the preferred source of animal protein [6]. Red jungle fowl (*Gallus gallus*) has been deemed as the wild ancestor of domestic chicken (*Gallus gallus domesticus*). The original chicken is the wild ancestor of domestic chicken [7]. Because of the pleasant tinnitus of "Camellia blossoming" when crowing, it is called Camellia chicken in Yunnan. Xishuangbanna Dai Autonomous Prefecture has abundant rainfall and sunshine, with an annual rainfall of 1136–1513 mm and an annual average temperature of 18.9 °C – 22.6 °C. Due to the warm and humid tropical rain forest climate, Xishuangbanna rich in biodiversity, which is very suitable for domestic chickens and their insect vectors. Avian haemosporidia is mainly transmitted by Diptera blood sucking insects such as mosquitoes, midges and simuliids [8, 9]. In poultry, avian haemosporidiosis can lead to clinical symptoms such as multiple organ injury, anemia and weight loss, which seriously affects the economic benefits of poultry breeding [10, 11]. If preventive treatment is not timely, it will lead to higher incidence rate and mortality [12].

Information about patterns of distribution of haemosporidians in poultry contributes to better prevention, control, and treatment of avian haemosporidiosis. However, up to date, there is limited study about avian haemosporidians infection in red jungle fowls (*Gallus gallus*). Therefore, the main objectives of the present study were to investigate the prevalence, molecular characterization, and associated risk factors of haemosporidians in red jungle fowl by using molecular biology methods and high throughput sequencing, evaluating the infection factors of haemosporidians in red jungle fowl using cross-sectional analysis.

## Methods

### Sample collection

With the help of the staff of Yunnan Province Center for Animal Disease Control and Prevention, Xishuangbanna Dai Autonomous Prefecture Technical Extension Station for Animal Husbandry and Veterinary Medicine, from November 2020 to May 2021, a total of 234 blood specimens were collected from red jungle fowl (*Gallus gallus*) which habitat in tea plantation, Jinghong City (21°27'~22°36'N, 100°25'~101°31'E), Yunnan Province, southwestern China. 112 samples of them were collected in 2020, and 122 samples were collected in 2021. A total of 103 samples were collected in winter (October to December) while 131 in summer (April to June). These domestic chickens were divided into two age groups: Juvenile, and Adult. Samples were divided into three groups according to body weight:  $\leq 0.5$  kg,

0.5-1.0 kg, and 1 kg. Each fresh blood specimen was randomly obtained from the inferior pterygoid vein of each apparently healthy fowl using the vacuum blood collection tube with anti-clotting agents such as EDTA. Then the vacuum blood collection tube contained approximately 2–4 ml individual animal blood samples were marked with animal sex, weight, age, sampling site, sampling time, and immediately kept on ice packs during transportation, stored at 4°C until used for staining and microscopic observation. Blood samples used for molecular analysis were stored in EDTA and maintained at – 80°C.

## Molecular analysis

The genomic DNA of each blood samples was extracted using the commercial DNA kit (Tiangen Bio-tech Co., Ltd, Beijing, China) according to the manufacturer's instruction. The extracted genomic DNA were stored at -20°C for further PCR analysis. The infection of avian haemosporidians in red jungle fowl (*Gallus gallus*) was detected by nested PCR amplifying for amplification of a 479 bp fragment of the mitochondrial cytochrome *b* gene (*cytb*) using primers and procedures described previously [13]. For the 1st PCR, the primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC – 3') were used. In the 2nd PCR, two primer pairs were applied: the primers HaemNF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemNR2 (5'-GCATTATCTGGATGTGATAATGGT-3'), and also HaemNFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemNR2L (5'-CATTATCTGGATGAGATAATGGIGC-3'). Amplification product was tested by running 2 µl of the 2nd PCR product on 1.5% agarose gel stained with SYBR Green I and visualized in an ultraviolet trans-illuminator (GDS-8000PC, GENE, USA). One negative control (nuclease-free water) and three positive controls were used to determine possible false amplifications.

## Bioinformatics, lineage identification and phylogenetic analysis

All positive secondary PCR products were purified and sequenced in Kunming Sangon Biotech (Shanghai) Co., Ltd. Sequences obtained were firstly proof read with their DNA peak-form graph using Chromas 2.6. By using MEGA X (Version 10.2.6, <https://www.megasoftware.net/>), the amplification products were aligned with most similar lineages according to the BLAST® result in MalAvi database (<http://130.235.244.92/Malavi/blast.html>) [5, 14]. Haplotypes were defined as new lineages if they differ by 1 bp from lineages deposited in the MalAvi database (<http://mbio-serv2.mbioe.kol.lu.se/Malavi>). The phylogenetic analysis was performed using the neighbor-joining (NJ) method with MEGA X, and the Kimura 2-parameter model was selected, 1,000 bootstrap replicates was applied in this study. The numbers at the nodes indicate the bootstrap support obtained by repeating the analysis 1000 times, and values above 50% are shown. Representative nucleotide sequences obtained in this study were deposited in the GenBank under accession numbers OM965002-OM965004 for avian haemosporidians.

## Statistical analysis

The prevalence and variation of avian haemosporidian parasites among different red jungle fowl (*Gallus gallus*) groups according to gender, age, weight, and sampling season were calculated by chi-square ( $\chi^2$ ) tests using SPSS 22.0 (IBM Inc., <https://www.ibm.com/cn-zh>), and were considered statistically

significant if the  $P$ -value  $< 0.05$ . The odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated and analyzed by using GraphPad Prism Version 8.02 for Windows (GraphPad Software, Inc., <https://www.graphpad.com/>).

## Results

# Prevalence of Avian Haemosporidium in Red Jungle Fowls

In the present study, haemosporidian parasites that belong to the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon* was detected in red jungle fowls (Table 1, 2, Fig. 1). The prevalence of avian haemosporidium in 234 blood samples from red jungle fowls in China was determined. As shown in Table 1, 175 avian haemosporidium DNA samples were detected amounting to 74.8% (175/234) overall prevalence of avian haemosporidium DNA in all blood samples.

Among them, 107 were positive for haemosporidium infection in adult fowls with the infection rate of 81.1% (107/132), while the infection rate of haemosporidium in juvenile fowls was 66.7% (68/102). Significant difference was observed between the two age groups ( $P = 0.012 < 0.05$ ,  $df = 1$ ,  $\chi^2 = 6.32$ ). The positive rate of blood samples collected in summer (80.9%, 106/131) was higher than in winter (67.0%, 69/103). According to Chi-square test, we identified the risk factors for the prevalence of avian haemosporidium in fowls as following: age (odds ratio, 0.47; 95% confidence interval [CI], 0.26 to 0.58;  $p = 0.012 < 0.05$ ), season (odds ratio, 2.09; 95% CI, 1.15 to 3.80;  $p = 0.015 < 0.05$ ).

The infection of avian haemosporidium can be divided into single infection and mixed infection in red jungle fowl (*Gallus gallus*) (Table 2). Of 175 blood samples that tested positive by the PCR technique, 153 (153/175, 87.4%) samples were single pathogen infections, of which 7 samples were *Haemoproteus* infections, 32 samples were *Leucocytozoon* infections, and 114 samples were *Plasmodium* infections. In addition, there were 22 (22/175, 12.6%) samples with mixed infections, with 16 samples infected with two pathogens and 6 samples infected with three pathogens.

## Molecular Characterization of avian haemosporidium

Molecular analysis revealed parasites belonging to three different genera of haemosporidians, *Haemoproteus*, *Plasmodium* and *Leucocytozoon* (Fig. 1). In this study, the three lineages of haemosporidians were clustered with their genetically most similar lineages of corresponding parasite genera. Our representative three lineage hGALGAL01, IGALGAL01, pGALGAL01 are more similar to *Haemoproteus enucleator*, *Leucocytozoon californicus*, *Plasmodium juxtannucleare* respectively (Fig. 1).

## Discussion

Avian haemosporidium is an extremely common problem all over the world, and responsible for anemia, acute tissue degeneration and depopulation in birds, especially for *Leucocytozoon* and *Plasmodium* [15, 16]. The diagnosis, prevention and control of avian haemosporidia are critical for poultry breeding,

domestic and wild bird protection. For avian haemosporidiosis, it can be diagnosed with clinical observation, combining morphological and molecular biology techniques from the veterinary standpoint. Relevant research reports can be used a mixture of trimethoprim and sulphaquinoxaline (TMP/SQX, ratio 1:3), with a wide spectrum of activity against bacteria and coccidia, and blood-induced *Plasmodium gallinaceum* malaria [17].

The traditional detection method of haemosporidium is carry out by observing the blood smears of birds under the microscope, identifying and classify them according to a series of morphological characteristics such as the size, shape, position and pigment arrangement of the parasite gametophyte or schizont (*Plasmodium* only) in the blood cells [8, 18]. Microscopic examination of blood smears requires experience and high quality of blood smears, and the sensitivity is limited, so false negative results occur frequently. In addition, microscopic examination is difficult to distinguish species with similar morphological characteristics (e.g., Plasmodium which has many hidden species). Microscopic examination often underestimates the diversity of haemosporidia. With the development of science and technology, molecular methods based on PCR (polymerase chain reaction) technology have gradually become popular, greatly improving the detection efficiency, sensitivity and precision [19].

In this research, the global prevalence of avian haemosporidia in red jungle fowls (*Gallus gallus*) was 74.8% (175/234), which was much higher than that of fighting cocks (*Gallus gallus*) from Thailand (20.8%, 52/250) [15], in domestic chicken (*Gallus gallus*) from Nan, Prachinburi, and Chachoengsao Provinces of Thailand (79.6%, 125/157) [20]; but it is lower than that in indigenous chickens from the North Central part of Nigeria (75.0%, 81/108) [21]. The prevalence of avian haemosporidia infection was higher in birds in tropical areas than in other areas, such as Jinghong City, Xishuangbanna belongs to the tropics. The reason for this may be the abundance of vegetation in tropical areas living with species of *Culicoides* and avian haemosporidia transmitted by biting midges [22, 23]. In addition, the reason for the prevalence variation is complicated, and many factors will affect the detection rate such as sampling time, age group, sampling number and geographic conditions [24]. In addition, similar to previous studies, the proportion of single infection was much higher than that of mixed infections [25, 26], and mixed infections showed multiple combinations [27, 28].

Avian haemosporidians has been detected in juvenile fowls and adult fowls with the infection ratio of 66.7% (68/102) and 81.1% (107/132) ( $P = 0.012 < 0.05$ ), respectively. In previous studies showed that infection rates were higher in young birds relative to adults, possibly due to the lower immune resistance in young birds [29, 30]. Juvenile and adult bird infected haematosporidia belong to distinct lineages, indicating that chicks got infection from non-parent birds [31]. Larger bare skins of young gulls make them accessible to the pathogen vectors more easily [32]. The weight of black-headed gulls did not appear to contribute significantly to *Haemoproteus* sp. infection. It is true that many studies have shown that different host-traits and abiotic factors are important determinants in a host-parasite interaction [33, 34]. Factors such as plant richness, vector species, temperature, and humidity in wild bird habitats contribute significantly to the prevalence and diversification of *Haemoproteus* sp. [35–38].

Avian haemosporidians in birds is genetic diversity [39, 40]. Representative *Haemoproteus* gene (accession no. OM965002) is closely related to *Haemoproteus* sp. in avian from India (99–100% similarities) (accession no EF380176.1) [40]. The *Plasmodium juxtannucleare* (accession no. OM965004) in this study is very similar to the strain from Thailand (accession no. KU248845.1). The lineage detected in the present study is new and may be a novel lineage from in red jungle fowls (*Gallus gallus*). We revealed that the known and novel lineage found in this study have biological invasion in China and can be transmitted to other birds.

## Conclusion

In the present study, the presence and assemblages of avian haemosporidians were identified in red jungle fowls (*Gallus gallus*) with different gender and age from Yunnan province, southwestern China. Avian haemosporidians belonging to the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. This study combined morphological and molecular phylogenetic analyses to identify avian haemosporidians in fowls, providing new information on the prevalence, epidemiology and geographical distribution of haemosporidian parasites circulating in fowls. Further whole mitochondrial genome analysis and morphology determination should be done to better evaluate gene diversity of haemosporidian parasites in diverse avian species. Up to now, this is the first records of avian haemosporidians infection in red jungle fowls (*Gallus gallus*) in China.

## Abbreviations

Cyt b: cytochrome b gene; ORs: odds ratios; CIs: confidence intervals; PCR: polymerase chain reaction

## Declarations

### Acknowledgements

We would like to thank Sangon Biotech (Shanghai) for technical assistance.

### Ethics approval

The protocol of the present study has been reviewed and approved by the Animal Ethical and Welfare Committee of Yunnan University. All blood samples were collected from red jungle fowls (*Gallus gallus*) after the permission of the poultry farmers and functional management departments without other authorities and all procedures is strictly in accordance with legal requirements of Animal Ethics Procedures and Guidelines of the People's Republic of China. All efforts were made to minimize suffering of fowls.

### Consent for publication

Not applicable.

## Availability of data and materials

The datasets supporting the findings of this article are included within the article. The data sets supporting the results of this article were deposited in the GenBank database (OM965002-OM965004).

## Competing interests

The authors declare that they have no competing interests. The co-author Prof Xing-Quan Zhu serves as the Subject Editor for the section “Parasite genetics, genomics and proteomics” of Parasites & Vectors.

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## Authors' contributions

ZL and QSL conceived and designed the study, and JJH critically revised the manuscript. ZL, YJZ performed the experiment, ZL and XXR analyzed the data and drafted the manuscript. ZL, BFD and NYH conducted the sample collection. JJH, FCZ, XQZ and QSL helped in the implementation of the study. All authors have read and agreed to the published version of the manuscript.

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## Tables

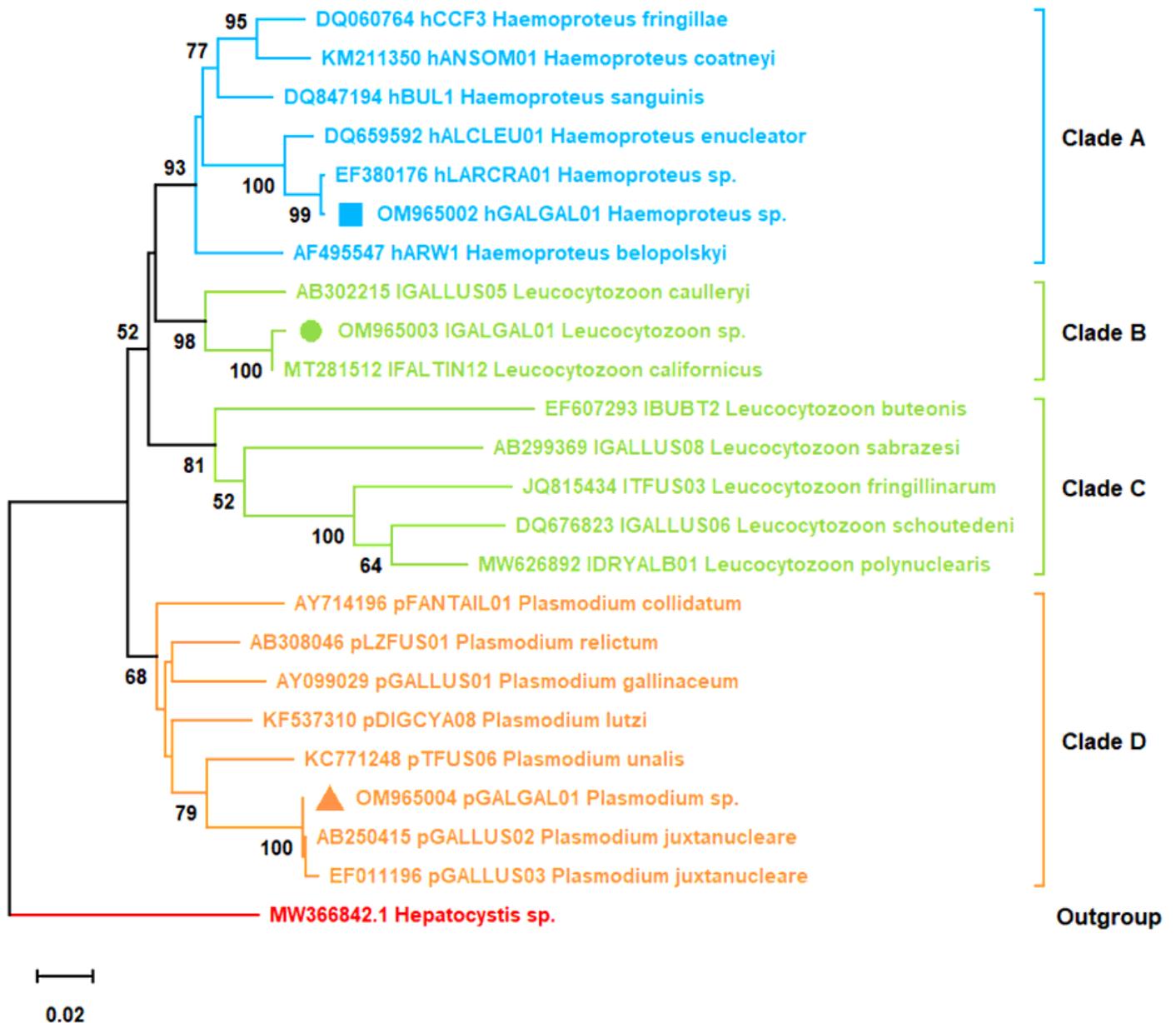
**Table 1** Prevalence of avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) in blood samples from red jungle fowl (*Gallus gallus*) determined by PCR, Yunnan Province, China, 2020-2021

Variable	No. positive / tested	Prevalence (95% CI)	Odds ratio (95% CI)	<i>P</i> * value
<b>Gender</b>				0.528
Female	123 / 167	73.7 (67.0-80.3)	Referent	
Male	52 / 67	77.6 (67.6-87.6)	0.81(0.41-1.58)	
<b>Age</b>				0.012
Juvenile	68 / 102	66.7 (57.5-75.8)	Referent	
Adult	107 / 132	81.1 (74.4-87.7)	0.47(0.26-0.58)	
<b>Weight</b>				0.149
≤0.5 kg	32 / 48	66.7 (53.3-80.0)	Referent	
0.5-1.0 kg	87 / 118	73.7 (65.8-81.7)	0.71(0.35-1.47)	
≥1 kg	56 / 68	82.4 (73.3-91.4)	0.43(0.18-1.02)	
<b>Seasons</b>				0.015
Summer	106 / 131	80.9 (74.2-87.6)	Reference	
Winter	69 / 103	67.0 (57.9-76.1)	2.09(1.15-3.80)	
<b>Total</b>	175 / 234	74.8 (69.2-80.5)		

**Table 2** Infection information, Parasite Species, and lineage of avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) in red jungle fowl (*Gallus gallus*) in Yunnan Province, China

Infection type	Parasite Genus	No. positive	Proportion %	Parasite Species	Lineage name	
Single infected	H	7	4.0	<i>H. enucleator</i>	hGALGAL01	
	L	32	18.3	<i>L. californicus</i>	IGALGAL01	
	P	114	65.1	<i>P. juxtannucleare</i>	pGALGAL01	
<b>Subtotal</b>		153	87.4			
Mixed infected	H, L	3	1.7	<i>H. enucleator</i>	hGALGAL01	
				<i>L. californicus</i>	IGALGAL01	
	H, P	3	1.7	<i>H. enucleator</i>	hGALGAL01	
				<i>P. juxtannucleare</i>	pGALGAL01	
	L, P	10	5.7	<i>L. californicus</i>	IGALGAL01	
				<i>P. juxtannucleare</i>	pGALGAL01	
	H, L, P	6	3.4	<i>H. enucleator</i>	hGALGAL01	
				<i>L. californicus</i>	IGALGAL01	
				<i>P. juxtannucleare</i>	pGALGAL01	
	<b>Subtotal</b>		22	12.6		
	<b>Total</b>		175 / 234			

## Figures



**Figure 1**

Phylogenetic tree of avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) based on *cytb* sequences.

Phylogenetic tree of avian haemosporidians based on *cytb* sequences. One lineage of *Hepatocystis sp.* was used as an outgroup. Parasite species names and GenBank accession numbers are provided in the tree. The parasite lineages reported in this study were marked by blue square, green dot, yellow triangle respectively. Bootstrap value is shown when value over than 50%.