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High prevalence and narrow host range of haemosporidian parasites in Godlewski's bunting (*Emberiza godlewskii*) in northern China

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ABSTRACT

Avian haemosporidian parasites are highly diverse, have a wide range of host specificity, and reveal diverse compatibility with regard to host range and geographical distribution. Therefore, understanding haemosporidian parasite diversity in different host species and different regions is crucial. A survey of the haemosporidian parasite in 186 Godlewski's buntings in Beijing was conducted to compare infection patterns between Godlewski's bunting, local passerines and the global avian host. High prevalence (88.7%) was found in the bunting and displayed annual stability during the research period. Most of the infections were caused by four dominant lineages, three of which were clustered with lineages of morphological species. In comparison with other lineages in local passerines, the dominant lineages were relative specialists. The findings suggest that the compatibility of dominant lineages in the bunting hosts may play important roles in high haemosporidian prevalence, and the narrow host range of the dominant lineages may be due to coevolution between the parasites and host species.

1. Introduction

Avian haemosporidians are cosmopolitan parasites transmitted by blood-sucking dipterans, which induce malaria-like infections in birds. With a high diversity of over 250 morphological species described from three genera [1], *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, a higher diversity has been revealed by the development of PCR-based diagnostic methods than by traditional blood smear examination [2,3]. The MalAvi database, which was established to collect mitochondrial cytochrome *b* (cyt *b*) sequences of avian malaria parasites [4], has so far recorded > 1300 unique lineages.

Most avian haemosporidian parasite research has been carried out in Europe and North America, leading to a substantial geographical bias with regard to the global diversity of these parasites [5]. Only a limited amount of PCR-based research has been used to survey avian haemosporidian parasites in China [6–8], and all of this research has focused on the parasite in host communities. Previous research conducted in Beijing, northern China, has discovered a high diversity and temporal dynamic of haemosporidian assemblages in passerines [6]. This research found that the yearly prevalence of *Plasmodium* and *Haemoproteus* fluctuated in opposite directions, and some abundant lineages did not occur every year [6]. However, the limited sample size of each avian species limits accurate reflection on the prevalence dynamics of haemosporidian parasites in certain host species.

Godlewski's bunting is a common resident species in Beijing, and high prevalence was detected in a pilot survey. This species, therefore, may be an important reservoir of local haemosporidian parasites. Most previous haemosporidian studies sampling old world buntings were conducted in Europe, and most revealed relatively high prevalence of the parasite (e.g. [9–13]). Only two studies have been conducted in east Asia, both in Japan, and they revealed, contrary to other research, extremely low prevalence [14,15]. In this research, a survey of avian haemosporidian parasite diversity in Godlewski's bunting in Beijing was conducted. Temporal stability of the parasite assemblage, prevalence over a two-year sampling period, and the relationship between lineage prevalence and host range were tested.

2. Material and Methods

2.1. Study site and sampling method

Godlewski's buntings were captured with mist nets between May and July 2014 and 2015 in Mentougou, Beijing, China (39° 57′ 54″ - 40° 4′ 15″ N, 115° 25′ 43″ - 116° 0′ 36″ E). Blood samples (ca. 40 μl) were taken from each bird by puncturing the alar vein, and samples were stored in ethanol at −40 °C.

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Table 1
Frequency, prevalence and lineage identification of haemosporidian parasites in Godlewski's bunting.

Genus	Haplotype	Frequency	Prevalence			MalAvi BLAST result	
			2014	2015	Two years	Lineage	Identity
Plasmodium	ALARV04	85	37.78%	48.23%	45.70%	ALARV04	100%
	PADOM02	60	26.67%	34.04%	32.26%	PADOM02	100%
	DENVID02	58	35.56%	29.79%	31.18%	DENVID02	100%
	DELURB5	2	0.00%	1.42%	1.08%	DELURB5	100%
	SGS1	1	2.22%	0.00%	0.54%	SGS1	100%
	EMGOD03	1	0.00%	0.71%	0.54%	PADOM02	99%
Haemoproteus	EMSPO01	5	4.44%	2.13%	2.69%	EMSPO01	100%
	EMGOD04	1	0.00%	0.71%	0.54%	FIPAR02	99%
Leucocytozoon	EMSPO05	36	24.44%	17.73%	19.35%	EMSPO05	100%
	BT2	7	2.22%	4.26%	3.76%	BT2	100%
	BT5	2	0.00%	1.42%	1.08%	BT5	100%
	EMGOD05	1	0.00%	0.71%	0.54%	EMSPO04	99%
	EMGOD06	2	0.00%	1.42%	1.08%	PYJOC02	98%
	EMGOD07	1	2.22%	0.00%	0.54%	COLBF01	92%
	EMGOD08	1	2.22%	0.00%	0.54%	COLBF01	92%

2.2. Parasite detection and identification

DNA was extracted from blood samples using the TIANamp Genomic DNA Kit (Tiangen Biotech Ltd., China). The mitochondrial cytochrome *b* (cyt *b*) gene of the haemosporidian parasite was amplified by nested PCR [16]. HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3′) were first used to amplify the cyt *b* gene of *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. The product of the first PCR was then used as a template for the second PCR. Two pairs of primers were employed for different taxa, with HaemF (5'-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3′) used for *Plasmodium* and *Haemoproteus*, and HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3′) and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3′) for *Leucocytozoon* [16].

The first PCR was performed in a 20 μl reaction mixture containing 10 μl 2× ExTaq premix (Takara, Japan), 0.4 μl of each primer, 7.2 μl of ddH_2O and 2 μl of the template DNA. The second PCR was conducted in a 40 μl reaction mixture with the same proportion of each component as the first reaction. ddH_2O was used as the template for negative control in each PCR to detect potential contamination. Three nested PCR repeats were conducted for each sample to avoid false-negative results. Infection of samples was determined by 2% agarose gels stained with SYBR Green I in an ultraviolet trans-illuminator (GDS-8000PC, GENE, USA), and amplified fragments were sequenced in both directions using an 3730XL automatic sequencer (ABI, USA).

Sequences were assembled using CodonCode Aligner 5.1 (CodonCode Corporation, USA). DNA chromatograms with multiple peaks were considered multiple infections. If three repeats of a multiple infection sample had one or more chromatograms without multiple peaks, these sequences were used to divide ambiguous sequences into two or more lineages. If all three repeats revealed multiple infections, the chromatogram was aligned with sequences confirmed from other samples to determine the exact combination [17].

Haemosporidian lineages were identified by comparing the 479-bp fragment of the cyt *b* gene with data from the MalAvi database. A new lineage was defined when the cyt *b* sequence had at least one nucleotide difference from lineages in the MalAvi database [4].

2.3. Phylogenetic analysis

Previous haemosporidian lineages of passerines in Beijing (GenBank No.KT757541 - KT757584) were employed to reconstruct the phylogenetic tree. A *Plasmodium falciparum* sequence (GenBank No. M76611.1) was used as the outgroup. Four lineages of morphological species, which are closely related to lineages in the present study (one lineage of *Plasmodium elongatum* (GRW06 [18]), and three of *Plasmodium relictum* (LZFUS01 [19], GRW04 [20] and GRW11 [21]) were employed for phylogenetic analysis. The most suitable nucleotide substitution model was selected using jModelTest2 [22] according to the Bayesian Information Criterion (BIC). BEAST v 1.8.0 [23] was used for phylogenetic reconstruction. In a total of 1×10^8 generations of the Markov chain, Monte Carlo (MCMC) was conducted and logged every 1×10^4 generations. The maximum credibility tree was searched by TreeAnnotator v1.8.0 utilizing a burn-in of the first 1000 trees.

2.4. Parasite community analysis

Annual variation of total prevalence was analyzed using Fisher's exact test, and a likelihood ratio test was used to assess whether annual variations of the parasite genus and lineage prevalence between the two years were significant. Prevalence of parasite lineages in this study and passerine community data from a previous study [6] were compared using Fisher's exact test.

The host range of local passerines was also compared with both dominant parasite lineages and rare lineages in Godlewski's bunting. Host ranges and prevalence of haemosporidian lineages in local passerines were extracted from Huang et al.[6], and global host ranges of the lineages were extracted from the MalAvi database v2.3.0 [4].

3. Results

In total, 186 Godlewski's buntings were examined (45 in 2014 and 141 in 2015). Of these, 165 (88.7%) were infected by haemosporidian parasites according to PCR-based diagnostics. Multiple infections were common, with 70 buntings (42.4%) infected by two lineages, 11 buntings (6.7%) by three lineages and two buntings (1.2%) by four lineages. *Plasmodium* had the highest prevalence (86.0%), followed by *Leucocytozoon* (26.3%) and *Haemoproteus* (3.2%).

In total, 15 cyt *b* lineages were identified in Godlewski's bunting (six *Plasmodium*, two *Haemoproteus*, and seven *Leucocytozoon*), four of which were not previously recorded in the MalAvi database (EMGOD03, EMGOD05, EMGOD07, and EMGOD08) (Table 1). Seven of the lineages in sympatric passerines have been reported in previous research [6], but three of these have been named differently here (EMGOD04 corresponding to AEGCAU03, EMGOD06 corresponding to PARUS64 and DENVID02 corresponding to EMGOD01) due to the assembling of longer sequences (479 bp) than in the previous research (432 bp) [6]. Three *Plasmodium* lineages (DENVID02, ALARV04, and PADOM02) and one *Leucocytozoon* lineage (EMSPO05) contributed most infections in Godlewski's buntings, while the prevalence of other lineages was below 4% (Table 1). No significant difference was found in haemosporidian

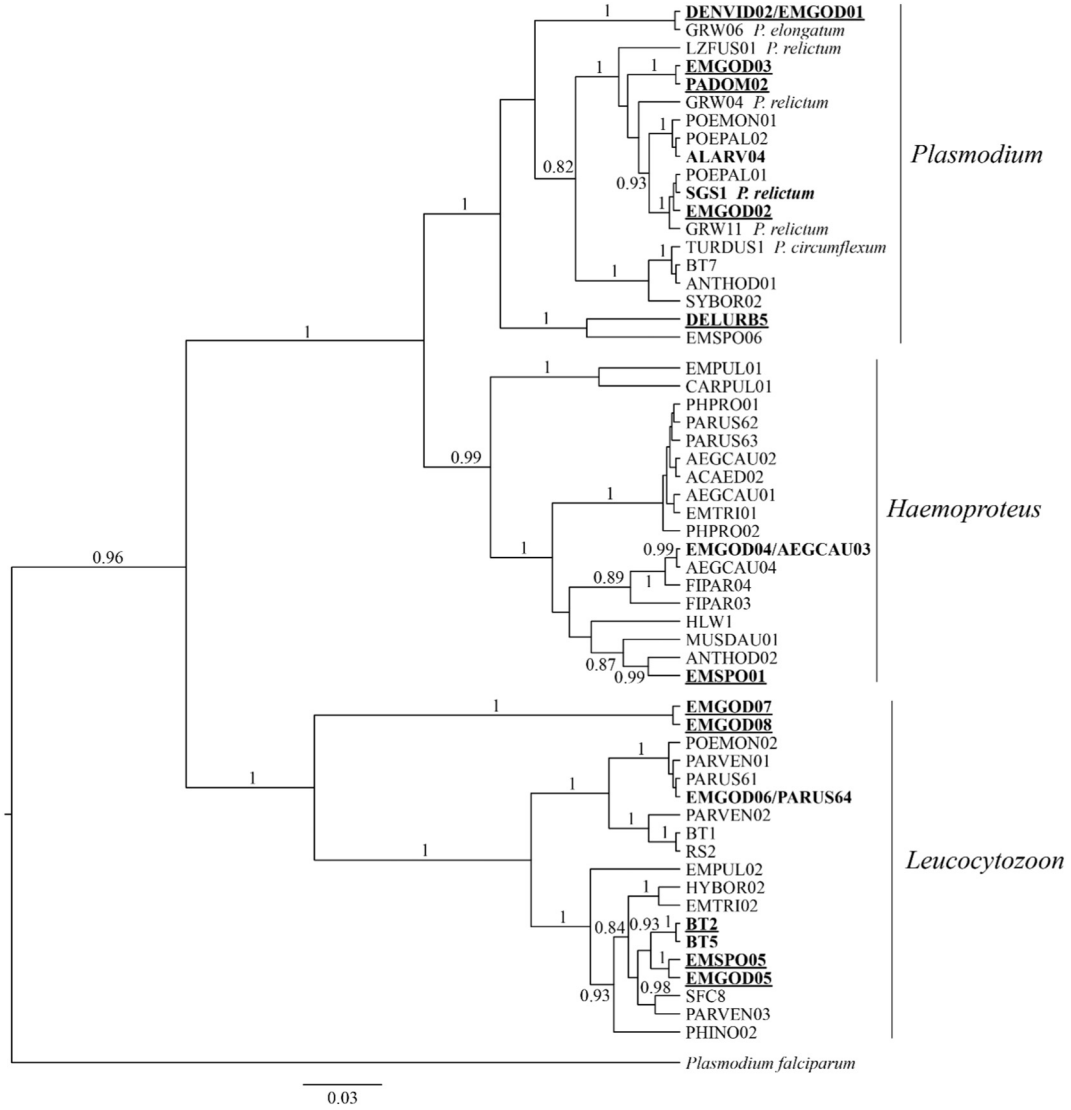

Fig. 1. Phylogenetic relationships of haemosporidian lineages of Godlewski's bunting and the local passerine community [6]. Estimated by Bayesian analysis of partial cyt *b* gene, analyzed under TN93 + Γ model. Posterior probabilities over 0.8 are indicated on branches. Bold: detected in Godlewski's bunting and other local passerines. Bold with underlining: only detected in Godlewski's bunting. Morphspecies names were labeled after lineages. Four lineages of morphospecies (GRW04, GRW06, GRW11 and LZFUS01) are employed for phylogenetic analysis.

prevalence between 2014 and 2015 (total, $P = .596$; genus, $\chi^2 = 0.390$, df = 2, $P = .823$; lineage, $\chi^2 = 17.031$, df = 14, $P = .255$).

The phylogenetic relationships of the lineages were confirmed by both MalAvi BLAST and the Bayesian tree (Fig. 1). Three dominant lineages of *Plasmodium* were divided into two clades (ALARV04 and PADOM02 in one clade, DENVID02 in another). DENVID02 was closely related to GRW06 (*P. elongatum*). All lineages of *P. relictum* and seven other lineages clustered in one clade, including four lineages of Godlewski's bunting (ALARV04, PADOM02, SGS1 and EMGOD03) (Fig. 1).

Within the *Leucocytozoon* clade, the four lineages which account for most *Leucocytozoon* infections (46 of 50 infections) clustered in one clade (Table 1, Fig.1), and three of these only infect the Godlewski's bunting. Two lineages (EMGOD07 and EMGOD08) formed a sister group to all other *Leucocytozoon* lineages with 8% genetic difference to the most closely-related lineage (COLBF01) in MalAvi (Fig. 1, Table 1).

The four dominant lineages in Godlewski's bunting showed a medium or narrow host range in the local passerine community (Supplementary Table 1). The prevalence of nine lineages showed significant differences between Godlewski's bunting and local passerines (Fisher's exact test, $P < .05$), with three (ALARV04, DENVID02, and EMSPO01) having a significantly higher prevalence in buntings, and three others (PADOM02, EMSPO05, and BT2) not previously detected in local passerines (Fig. 2). Most lineages with a broad host range (e.g. SGS1, ACAED02, and HLW1) displayed lower prevalence or complete absence in Godlewski's bunting (Fig. 2, Supplementary Table 1).

4. Discussion

4.1. Stably high prevalence by a few dominant lineages

The prevalence of haemosporidian parasites in Godlewski's bunting was extremely high (88.7%), displaying a much higher prevalence than in any other passerine species sampled in this area (at least 5 samples) [6], and a much higher prevalence than has been previously reported for other *Emberiza* species (e.g. [9–13,24,25]).

The prevalence of certain haemosporidian lineages is dependent on the proportion of susceptible individuals in a host population and the probability of exposure by hosts to the parasites [26]. Four dominant lineages contributed to > 90% of infections in buntings over two consecutive years, indicating that Godlewski's bunting is a compatible host

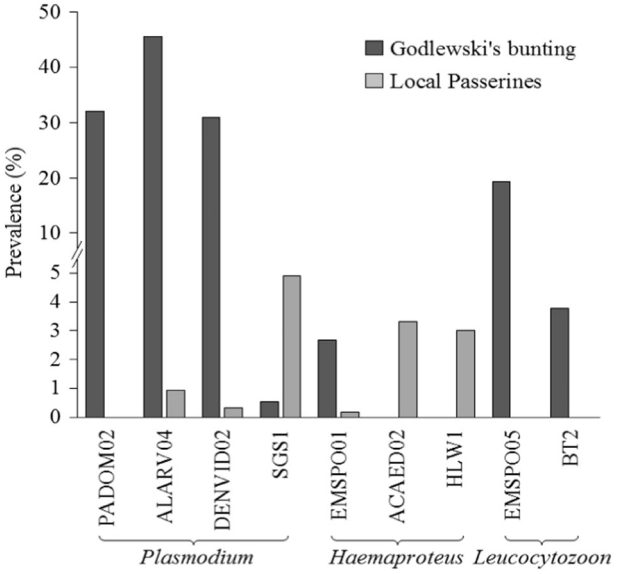

Fig. 2. Prevalence comparisons of lineages with significant difference between Godlewski's bunting and the local passerines [6] (Fisher's exact test $P < .05$).

for these lineages, and the proportion of susceptible individuals of these lineages should be high in Godlewski's bunting populations [26]. The encounter probability of these dominant haemosporidian lineages is dependent on vector density in the host's habitat [27–29]. Godlewski's buntings nest in shrubs and usually forage near the ground, and further research is recommended to investigate the vectors of the haemosporidian parasite in Godlewski's bunting habitat, especially vectors of the dominant parasite lineages.

Comparing yearly fluctuating haemosporidian prevalence of local passerines at both genus and lineage level [6], parasite prevalence of Godlewski's bunting revealed more stability during the two-year sample period. If parasites are also stable between years in other sympatric passerine species (e.g. [30,31]), prevalence fluctuations in a host community may result from turnover or population variation of host species within the community. However, a two-year sample is insufficient to determine the annual prevalence variation of haemosporidian parasites, and long term sampling is necessary for future monitoring.

Moreover, the high prevalence in some lineages of Godlewski's bunting implies that this host species can serve as a reservoir for parasites that facilitate pathogen transmission to other potential susceptible hosts, as other research has discovered [32–34]. Previous research revealed that these dominant lineages are uncommon in local passerines. However, the sample size of some bird species was limited, especially with regard to some species closely related to Godlewski's bunting, such as the meadow bunting (*Emberiza cioides*) [6]. Therefore, it is important to enlarge the sample size of other sympatric bird species in order to understand the transmission of these haemosporidian lineages.

4.2. Host specificity and distribution of haemosporidian parasites

The host ranges of parasite lineages in Godlewski's bunting exhibited great variation. Among all haemosporidian lineages in the local passerine community, SGS1 (*P. relictum*) is the most cosmopolitan lineage and has been found in > 100 bird species of 11 orders (MalAvi V2.3.0, [4]). In addition, it has the widest host range in the local passerine community [6] (Table 1). However, SGS1 was extremely rare in surveyed Godlewski's buntings, with only one individual infected. Similar phenomena were also observed in other haemosporidian generalists at both local and global levels (eg. BT7, TURDUS1, ACAED02, HLW1, details in Supplementary Table 1) [4,6].

The global distributed lineage PADOM02 has infected 14 bird species (MalAvi V2.3.0, [4]), but had not been previously recorded in local passerines [6]. ALARV04 was found in eight host species from Asia and Oceania (MalAvi V2.3.0, [4]) (Supplementary Table 1). Although the local host range of ALARV04 was broadest within four dominant lineages, this is still much narrower than the local generalist [6] (Supplementary Table 1). The above two lineages clustered with four lineages of *P. relictum* and five others (Fig. 1). The largest genetic differentiation within this clade was 3.13% (lower than 5%), and the clade divided with other clades with strong support (Fig. 1). It can, therefore, be inferred that ALARV04 and PADOM02 may relate to *P. relictum* [3,35]. Morphological evidence is required in order to confirm this hypothesis.

Previously, DENVID02 had only been found in the white-faced whistling duck (*Dendrocygna viduata*) in Brazil [36]. These two distant host species with isolated geographic distribution of DENVID02 may be due to a monitoring gap between these two areas. Meanwhile, the BLAST result of DENVID02 shows high identity (only one base-pair difference) with the cosmopolitan lineage GRW06, which has a global distribution range [37–39]. This suggests the possibility that the identical DENVID02 lineages in the two separate sites may have evolved independently by mutation from GRW06.

EMSPO05 was the most abundant *Leucocytozoon* found in the Godlewski's bunting. It has previously only been found in the black-faced bunting (*Emberiza spodocephala*) in Russia (Palinauskas et al. unpublished) and the common rosefinch (*Carpodacus erythrinus*) in the Czech Republic [40]. The subspecies *E. s. spodocephala* of the black-faced bunting, which reproduces in Siberia, regularly migrates through northern China (including Beijing) to southern and eastern China [41,42] and is phylogenetically related to Godlewski's bunting [43], which may give the parasite a chance for transmission. EMSPO05 has not be found in local passerines, but its local host range remains unclear, as passerines have yet to be tested by specific primers for *Leucocytozoon* [6,16]. A similar phenomenon was also found in EMSPO01, which was only detected in Godlewski's bunting from Beijing and black-faced bunting from Russia (Palinauskas et al. unpublished). EMSPO01 was the most frequent *Haemoproteus* lineage in Godlewski's bunting, but it remains much rarer than dominant lineages.

Within local haemosporidian lineages, *Leucocytozoon* lineages in Godlewski's bunting have stronger host specificity than *Plasmodium*. Lineages of most infections of *Leucocytozoon* (EMSPO05, BT2, BT5, and EMGOD05) were clustered together and three of these were lineages locally specific to Godlewski's bunting. Nevertheless, dominant lineages of *Plasmodium* were divided into different clades, as were other rare *Plasmodium* lineages (Table 1, Fig. 1). This difference of host specificity between *Plasmodium* and *Leucocytozoon* is consistent with the findings of previous research [12].

4.3. Specialists preserved higher prevalence?

This research indicates that dominant lineages in Godlewski's buntings have relatively small host ranges. Meanwhile, most parasites with a broad host range have lower prevalence than specialists (Fig. 2, Supplementary Table 1). Godlewski's bunting is a local resident bird and, therefore, can only become infected by parasites with local transmission routes. Long-term coevolution between Godlewski's bunting and local parasites may increase host specificity with compatible parasites and may also reduce susceptibility to exotic parasites which transmit from migratory birds [44].

However, if the results of this research are found to be common in other local species, this phenomenon could provide empirical evidence for the trade-off hypothesis of the abundance–occupancy relationship, which predicts that specialist parasites should archive higher prevalence than generalist parasites [17,45]. Further research of more taxa and larger sample sizes are required to test this hypothesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.09.004.

Data accessibility

Lineage data has been submitted to the public MalAvi Database. Sequence data from novel lineages (EMGOD03, EMGOD05, EMGOD07, and EMGOD08) will be deposited in GenBank.

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