

Occurrence of haemosporidian parasites in the paddyfield warbler, *Acrocephalus agricola* (Passeriformes, Sylviidae)

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Abstract

The blood parasite diversity was studied in paddyfield warblers (*Acrocephalus agricola*) breeding in NE Bulgaria, SW Russia and S. Kazakhstan. Nine cytochrome b gene lineages were recorded, 4 belonging to *Haemoproteus* spp. and 5 to *Plasmodium* spp. The overall prevalence of haemosporidians was 33.3%. The composition of parasites varied geographically, with six lineages recorded in Russia, five lineages in Bulgaria and two lineages in Kazakhstan. Two lineages are described for the first time, i.e. ACAGR1 (belonging to *Plasmodium* sp. and recorded from a single bird in Russia) and ACAGR2 (belonging to *Haemoproteus* sp., recorded from Bulgaria and Russia). The latter lineage is the most widespread parasite in the Bulgarian population, scarce in Russia and absent in Kazakhstan. It is supposed that ACAGR2 has originated from the widespread lineage ACDUM1 differing from it by a single nucleotide. One lineage only (ACDUM2) occurs in all the three populations studied and is a non-specific parasite known from various passerines. Six of the registered lineages have been found in a single population of *A. agricola* and also represent non-specific parasites occurring in a wide range of passerine birds. Their records in *A. agricola* may indicate the high transmission rate of these parasites in the habitats where this host co-occurs with other passerines. The variation of the composition of the haemosporidian parasite communities through the breeding range of *A. agricola* makes up heterogeneous selection pressures that may drive intraspecific variation in important life-history traits.

Keywords

Plasmodium, *Haemoproteus*, cytochrome b lineages, parasite community, geographical variation

Introduction

Data on haemosporidian parasites of migratory birds have been limited geographically, with research principally concentrated on the European-African migratory system and mainly to the breeding grounds in Western Europe. Very little information exists on the post-glacial range expansion and migratory strategies of avian host species wintering in India and SE Asia. It can be expected that there are essential differences between the blood-parasite composition emerged in migratory species wintering in Africa and that in birds wintering in South Asia due to extensive glaciation of Europe during the last glacial advance (in contrast to Asia).

The parasite community is a biotic factor that may cause varying selective regimes over wide areas (Valkiūnas 2001;

Reullier *et al.* 2006). Recent work on avian malaria parasites has revealed an unexpectedly rich fauna (Bensch *et al.* 2000, 2004; Waldenström *et al.* 2002, 2004). The avian haemosporidian community may differ between regions and can thus force an important heterogeneous selection pressure during population expansions (Valkiūnas and Iezhova 2001; Westerdahl *et al.* 2005).

The paddyfield warbler (*Acrocephalus agricola*) has a mosaic distribution in the Palaearctic, from NE Bulgaria in the west to NW Mongolia in the east (Voinstvenskiy 1960; Paspaleva and Talpeanu 1980). Its breeding range (Fig. 1) has recently expanded westwards (Cramp 1992) and in Bulgaria this species was first recorded in 1968 (Dontschev 1970, Nadler and Ihle 1988). Interestingly, the birds from the most western point of the breeding area (Durankulak Lake, NE Bulgaria) winter in

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the Indian Peninsula; thus, they must fly much longer distances to the wintering grounds than eastern populations. In autumn, Bulgarian paddyfield warblers circumvent the Black Sea along its northern coast following the historical expansion route of the species (P. Zehndjiev, unpublished data). The degree of genetic differentiation and gene flow of the Bulgarian population showed that there was no divergence (as revealed on the basis of a fragment of the mitochondrial control region and five microsatellite loci) between Bulgarian and Russian populations whereas a population from Kazakhstan differed significantly from the European populations. Furthermore, gene flow analyses confirmed the observation that the westwards expansion of the breeding range was recent (P. Zehndjiev, unpublished data).

In the present study, we examine the composition of pigmented haemosporidians in paddyfield warbler populations in NE Bulgaria, SW Russia and S. Kazakhstan. Previous studies describing haemosporidians in this host species are lacking. We were further interested in comparing the composition of haemosporidians between the newly established territories with the core area of the species distribution, since differences in composition may be expected due to isolation of the populations or due to transfer of lineages from other host species in the novel range.

Materials and methods

Population samples

We mist-netted paddyfield warblers and collected a blood sample (5–20 μ l) from the wing or tarsus veins. Samples were stored in SET-buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) at ambient temperature in the field and later at -20°C . Blood films were prepared on ready-to-use glass slides and were air-dried within 5–15 sec after their preparation. Smears were fixed in absolute methanol for 4 min on the day of their preparation. In the laboratory, the blood films were stained in a 10% working solution of a commercially purchased stock solution of Giemsa's stain, pH 7.0–7.2, at $18-20^{\circ}\text{C}$ for 1 h (Valkiūnas *et al.* 2008).

In total, we collected 138 samples at one site in Bulgaria, four sites in Russia and one site in Kazakhstan (Table I). The Bulgarian and Russian birds were sampled during the breed-

Table I. Collecting sites of the paddyfield warbler (*Acrocephalus agricola*) and number of samples tested for the presence of haemosporidians

Collecting sites	Samples
Bulgaria (Durankulak, 43°40'N/28°32'E)	57
Russia (Solyanka, 50°49'N/47°05'E)	34
Russia (Algay, 50°03'N/48°14'E)	15
Russia (Steppe Liman, 50°43'N/46°27'E)	16
Russia (Bobrovka, 51°31'N/49°15'E)	7
Kazakhstan (Balkhash, 46°51'N/74°56'E)	9

ing seasons of 2005 and 2006. Six samples from Kazakhstan (from the spring of 2001) from the collection of the Molecular Ecology Laboratory, University of Lund, were used; three other samples of breeding birds from Kazakhstan were provided by Dr. Petr Procházka (Institute of Vertebrate Biology, Brno, Czech Republic).

DNA analyses

For all individuals, except the 3 samples from Balkhash Lake in Kazakhstan, total DNA was extracted from whole blood (5–50 μ l) stored in SET-buffer (500 μ l) by a standard proteinase K and ammonium acetate protocol (Richardson *et al.* 2001). Three samples from Kazakhstan (blood in ethanol) were first dried, then resolved in SET-buffer and extracted according to the protocol used for the rest of the samples. Genomic DNA extracts were diluted to a working concentration of 25 ng/ μ l.

Parasites identification

Diluted genomic DNA was used as the template in polymerase chain reaction (PCR) assays for detection of parasites using primers and temperature profiles as in Hellgren *et al.* (2004). Positive or negative samples were seen as presence or absence of bands on a 2% agarose gel using 1.5 μ l of the final PCR product. Samples that showed positive amplification were sequenced using the procedures described by Bensch *et al.* (2000). Amplified fragments were sequenced from the 5' end with the primer HaemF. The obtained sequences of 479 bp of mitochondrial cytochrome *b* gene were edited, aligned and compared in a sequence identity matrix using the program BioEdit (Hall 1999). All unique lineages, i.e. sequences differing by one or more nucleotide difference, were sequenced in the reversed direction with the complementary primer. PCR amplifications from birds with mixed infections observed as “double base calling” in the electropherograms were considered as mixed infections. These samples were excluded from our analysis. The obtained sequences were matched and named according to the MalAvi database (Bensch *et al.* 2009). The sequences of the two new lineages were deposited in GenBank (accession numbers FJ861321 and FJ861322).

All PCR positive samples were confirmed by microscopy using an Olympus BX51 light microscope equipped with an Olympus DP12 digital camera and imaging software DP-SOFT. Blood films were examined for 10–15 min at low magnification ($\times 400$), and then at least 100 fields were studied at high magnification ($\times 1,000$) (Valkiūnas *et al.* 2008).

Statistical analyses

We used the software MEGA, version 2.1 (Kumar *et al.* 2004), to generate a neighbour-joining trees with a Kimura 2-parameter distance matrix. Trees were rooted in *Plasmodium falciparum* and *Haemoproteus columbae*. We used 500 replicates to estimate bootstrap support.

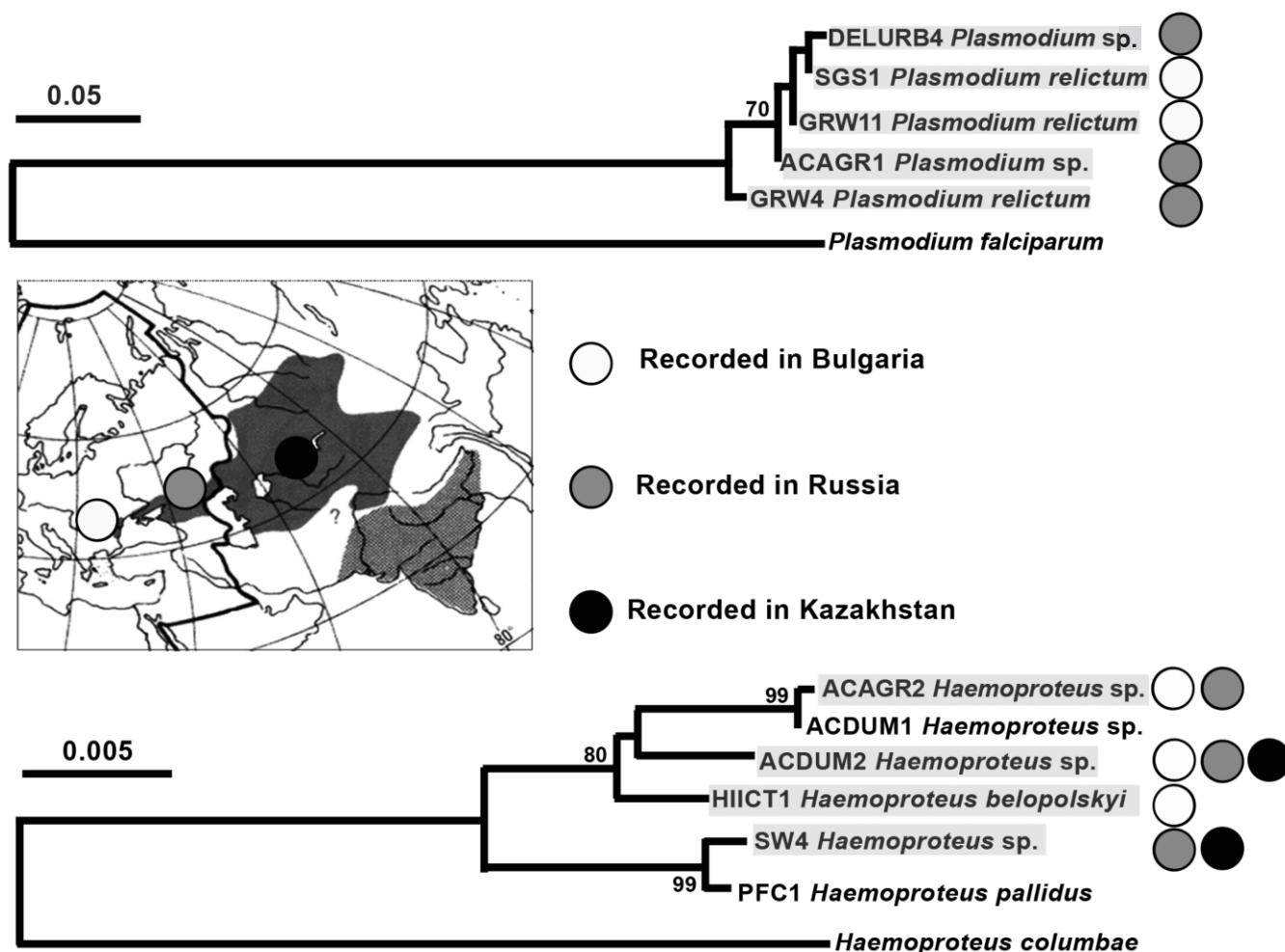


Fig. 1. Occurrence of the cytochrome *b* lineages of *Haemoproteus* spp. and *Plasmodium* spp. in paddyfield warblers (*Acrocephalus agricola*). Lineages recorded in the course of the present study are highlighted in grey. The phylogenetic trees were constructed with the neighbour joining method with Kimura 2-parameter distance. Bootstrap values (>70%) are shown. Circles indicate sampling sites (white – Bulgaria, grey – Russia, black – Kazakhstan). Inset: geographical range of the paddyfield warbler (after Cramp 1992)

The sequence divergence between the different lineages (Table III) was calculated with the use of a Jukes-Cantor model of substitution, with all substitutions weighted equally, implemented in the program MEGA 4.0 (Kumar *et al.* 2004).

Results

In a set of samples of 138 paddyfield warblers from Bulgaria (57), Russia (72) and Kazakhstan (9), we encountered 46 birds

Table II. Distribution of the cytochrome *b* lineages of *Haemoproteus* spp. and *Plasmodium* spp. and the number of birds infected with a single haemosporidian lineage along the three sampling sites of the paddyfield warbler (*Acrocephalus agricola*)

Location	<i>Haemoproteus</i> spp.				<i>Plasmodium</i> spp.					Total
	ACAGR2	ACDUM2	HIICT1	SW4	GRW11	GRW4	DELURB4	SGS1	ACAGR1	
Bulgaria (N=57)	7	4	1		1			3		16
Russia (N=72)	2	5		2		4	2		1	16
Kazakhstan (N=9)		4		1						5
Total (N=138)	9	13	1	3	1	4	2	3	1	37

Table III. Sequence divergence (in percentage) between 12 mitochondrial cytochrome *b* gene lineages of avian *Plasmodium* spp. and *Haemoproteus* spp. found in the paddyfield warbler (*Acrocephalus agricola*) and several related lineages (presented in the phylogenetic trees)

Lineage	H_ACAGR2	H_ACDUM1	H_ACDUM2	H_HIICT1	H_SW4	H_PFC1	H_columbae	P_DELURB4	P_ACAGR1	P_GRW4	P_GRW11	P_SGS1
H_ACDUM1	0.2											
H_ACDUM2	3.3	3.19										
H_HIICT1	3.7	3.5	2.5									
H_SW4	7.5	7.2	5.6	6.7								
H_PFC1	7.2	7	5.3	6.1	0.9							
H_columbae	20.6	20.2	22.5	19	19.8	20.8						
P_DELURB4	14.3	14	12.8	11.8	11.1	9.7	21.8					
P_ACAGR1	14.7	14.3	12.7	12.2	10.7	9.3	21.8	0.7				
P_GRW4	14.2	13.9	11.6	11.8	9.7	8.4	22.9	2.2	2			
P_GRW11	14.3	14	12.3	11.8	10.7	9.3	21.8	0.7	0.4	2		
P_SGS1	14.7	14.3	12.7	12.2	11	9.6	22.2	0.4	0.2	2.2	0.2	
<i>P. falciplarum</i>	42.8	42.1	37.7	40.3	44.3	42.3	58	36.5	34.3	33.6	34.3	34.9

infected with haemosporidians (12 infected with *Plasmodium* spp., 25 with *Haemoproteus* spp. and 9 with multiple infections). Thus, the overall prevalence of haemosporidian parasites was 33.3%. Nine lineages were identified (Fig. 1).

The prevalence in Bulgaria and Russia was almost equal, i.e. 28% (belonging to 5 lineages) and 22% (6 lineages), respectively. The prevalence in the samples from Kazakhstan was 55% representing two lineages of *Haemoproteus* only. The most common haemosporidian infections in Bulgaria were those with the *Haemoproteus* lineage ACAGR2 that is described for the first time in the present study. This lineage was also found in one first-year bird indicating local transmission of the parasite in the European breeding range. The closely related lineage ACDUM2 (*Haemoproteus* sp.) occurred in all the three populations studied (Fig. 1). The lineage HIICT1 was found in only one Bulgarian bird.

The lineages of *Plasmodium* spp. (GRW4, GRW11, SGS1, ACAGR1, DELURB4) were found only in Russian and Bulgarian birds (Fig. 1). The *Haemoproteus* lineage SW4 was found only in birds breeding in Kazakhstan and Russia. The observed diversity of parasites in the sampled populations was similar in Bulgaria and Russia, and substantially lower in Kazakhstan (Fig. 1).

Two of the cytochrome *b* lineages detected by us, i.e. ACAGR1 (representing *Plasmodium* sp. from Russia) and ACAGR2 (*Haemoproteus* sp. from Bulgaria and Russia), are recorded for the first time by the present study.

Discussion

Expanding populations encounter a wide variety of unoccupied habitats with varying selection regimes over wide areas, which can drive diversification in comparatively short periods. Such selection regime can be due to the parasite community (Valkiūnas 2001, Reullier *et al.* 2006). Recent work on avian haemosporidians has demonstrated a surprisingly diverse fauna (Bensch *et al.* 2000, 2004; Waldenström *et al.* 2002, 2004), with parasite communities differing between regions (Valkiūnas and Iezhova 2001; Bensch and Åkesson 2003; Westerdahl *et al.* 2005). In the present study, it is of interest to compare the composition of haemosporidians between the newly-established territories in Bulgaria with the core area of the breeding range (Russia and Kazakhstan), since differences may be expected due to both assimilation of lineages occurring in other host species in colonised areas and the evolution of novel lineages in isolated populations. We are limited by the small sample size (especially from Kazakhstan) and the variation observed between regions may in part reflect sampling error. However, acknowledging these shortcomings, some patterns are still worth pointing out.

The new lineage ACAGR2 is common in Bulgaria, scarce in Russia and absent in Kazakhstan. This pattern may have resulted from a transfer from another host species breeding in sympatry with the paddyfield warbler in the newly-colonised

breeding areas in the western parts of the range. Alternatively, it may be a newly-derived form originating from the common ACDUM1 (a common lineage in passerines, see Bensch *et al.* 2009), which differs from ACAGR2 by only one nucleotide (Fig. 1). It can be supposed that the evolvement of the new lineage has happened in parallel with the westwards expansion of the breeding range of the paddyfield warblers. This explanation is indirectly supported by the fact that ACAGR2 is not found in any other host species in Bulgaria despite extensive surveys of haemosporidian infections in this country (Valkiūnas *et al.* 2007, Zehindjiev *et al.* 2008). If the latter hypothesis will be confirmed by further studies, then the lineage ACAGR2 will be proved as an oioxenous parasite (i.e., specific to a single host species) of *Acrocephalus agricola*.

The other new lineage discovered by the present study, ACAGR1, has been recorded from a single bird only. On the basis of the present data, it is impossible to make any suppositions on its host specificity and size of its geographical range.

The lack of *Plasmodium* spp. in Kazakhstan is likely to be due to the small sample size but abundance of these parasites in Russia and Bulgaria, especially the lineages GRW4 and DELURB4 known as parasites of tropical transmission (Beadell *et al.* 2006, Palinauskas *et al.* 2007) may indicate different over-wintering grounds for these populations, e.g. in the western and eastern parts of the Indian Peninsula.

The parasite community is almost as rich in the newly-colonised range in Bulgaria as in the pooled sample from Russia and Kazakhstan. However, the comparison of the composition of lineages exhibits that only ACDUM2 occurs in all the three examined populations. Two other lineages, ACAGR2 and SW4, are each recorded in two populations; however, the latter has not been found in the westernmost breeding population of *A. agricola* in Bulgaria. The remaining six lineages are registered in one population only; they are known from other passerine birds (Bensch *et al.* 2009). Their present records in paddyfield warbler populations might be an indication for the high transmission rate of these parasites in the habitats where *A. agricola* co-occurs with other passerine hosts.

Various features of migratory strategies of bird species such as timing, orientation and flight distance have been documented to evolve rapidly in nature (Berthold *et al.* 1992, Able and Belthoff 1998). Rapid adaptive evolution of complex characteristics forming migratory strategies has been demonstrated in *Sylvia atricapilla* (Perez-Tris *et al.* 2004). Similarly, the paddyfield warbler has evolved a unique migratory program during the recent westwards expansion along the Black Sea to Europe (P. Zehindjiev, unpublished data). Considerable changes in morphology, behaviour and physiology may take place in a few generations (Losos *et al.* 1997, Orr and Smith 1998). It remains to be studied to what extent the rapid changes in the orientation behaviour of the paddyfield warbler may have been paralleled by other life-history adaptations such as resistance to blood parasites forming highly-divergent populations across the breeding range.

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