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Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds

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A fragment of the mitochondrial cytochrome *b* gene of avian malaria (genera *Haemoproteus* and *Plasmodium*) was amplified from blood samples of 12 species of passerine birds from the genera *Acrocephalus*, *Phylloscopus* and *Parus*. By sequencing 478 nucleotides of the obtained fragments, we found 17 different mitochondrial haplotypes of *Haemoproteus* or *Plasmodium* among the 12 bird species investigated. Only one out of the 17 haplotypes was found in more than one host species, this exception being a haplotype detected in both blue tits (*Parus caeruleus*) and great tits (*Parus major*). The phylogenetic tree which was constructed grouped the sequences into two clades, most probably representing *Haemoproteus* and *Plasmodium*, respectively. We found two to four different parasite mitochondrial DNA (mtDNA) haplotypes in four bird species. The phylogenetic tree obtained from the mtDNA of the parasites matched the phylogenetic tree of the bird hosts poorly. For example, the two tit species and the willow warbler (*Phylloscopus trochilus*) carried parasites differing by only 0.6% sequence divergence, suggesting that *Haemoproteus* shift both between species within the same genus and also between species in different families. Hence, host shifts seem to have occurred repeatedly in this parasite–host system. We discuss this in terms of the possible evolutionary consequences for these bird species.

Keywords: *Acrocephalus*; *Phylloscopus*; *Parus*; host–parasite coevolution; cytochrome *b*; malaria

1. INTRODUCTION

Studies of parasite–host interactions have revealed many of the most sophisticated examples of evolution, including adaptive manipulation of host behaviour (Lafferty & Morris 1996) and host sex (Hurst *et al.* 1993; Vance 1996). In birds, the prevalence of haematozoan blood parasites has been used to examine hypotheses of sexual selection (Hamilton & Zuk 1982; Read 1987; Zuk 1991), immunocompetence (McCurdy *et al.* 1998; Nordling *et al.* 1998) and the costs of reproduction (Norris *et al.* 1994; Richner *et al.* 1995; Wiehn *et al.* 1999). The most frequently studied avian blood parasites belong to the genera *Haemoproteus* and *Plasmodium* (Atkinson & Van Riper 1991).

Earlier morphological studies have suggested that *Haemoproteus* species are more likely to be host specialists than *Plasmodium* species (Atkinson & Van Riper 1991). However, the extent to which closely related bird species share the same or closely related *Haemoproteus* and *Plasmodium* species is virtually unknown. Restriction to a single host indicates that a parasite and its avian host have been associated over much of their evolutionary history (Hoberg *et al.* 1997). In contrast, the occurrence of one species of parasite on several host species suggests that host shifts occur frequently. Because host shifts are often associated with a change in virulence (Toft & Karter 1990), these blood parasites may play a complex role in the evolution of their avian hosts.

Many population studies of birds have found no or only marginal fitness effects of *Haemoproteus* on their bird hosts (Weatherhead 1990; Dale *et al.* 1996). When a parasite invades a new host, its virulence may increase or decrease (Bull 1994). The conventional view holds that parasites are initially more virulent than after several generations of exposure to a given host (Toft & Karter 1990). A well-known example supporting this view comes from studies of the introduction of avian malaria (*Plasmodium*) to native Hawaiian bird species (Van Riper *et al.* 1986). Reduced pathogenicity results from selection on the immune system of the host as well as selection on the parasite in order to reduce its virulence and, thereby, increase transmission between host individuals. Alternatively, Ewald (1983) suggested that the virulence of many parasites (particularly vector-transmitted parasites such as *Plasmodium* and *Haemoproteus*) may increase due to selection among competing parasite lineages within host individuals. For vector-transmitted parasites, high virulence may increase transmission, assuming that infected hosts become immobilized and, hence, more easily explored by the vector.

The extent of host specificity and host shifts among a group of parasites can be directly tested by comparing the match between the phylogenetic trees of the parasites and their hosts (Brooks 1988). A close match would suggest strong host specialization and co-speciation. Alternatively, a poor match would imply that host shifts have happened repeatedly over evolutionary time and that potential host species are at substantial risk of being invaded by novel parasites, which could affect the

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population size and subsequent evolution (Toft & Karter 1990; Newton 1998).

In the present study, we used the polymerase chain reaction (PCR) for amplifying and sequencing a portion of the cytochrome *b* gene of the mitochondrial DNA (mtDNA) of *Haemoproteus* and *Plasmodium* parasites in blood samples taken from 12 species of passerine birds of the genera *Acrocephalus*, *Phylloscopus* and *Parus*. We chose to study these genera because their species phylogenies have been resolved (Richman & Price 1992; Helbig & Seibold 1999) and much of the ecological research on *Haemoproteus* or *Plasmodium* has been done in *Parus* (e.g. Norris *et al.* 1994; Richner *et al.* 1995). We used the *Haemoproteus/Plasmodium* sequences retrieved from the 12 bird species to ask (i) whether the different bird species have unique blood parasites, (ii) to what extent one bird species can be infected by different parasite species, and (iii) whether the phylogenetic tree of the parasites matches the phylogenetic tree of their bird hosts.

2. METHODS

We used published sequences (Escalante *et al.* 1998) of the cytochrome *b* genes of *Plasmodium* and *Haemoproteus* in order to locate conserved regions. We used ClustalW (Thompson *et al.* 1994) for aligning these sequences against the cytochrome *b* sequences of two *Acrocephalus* warblers (GenBank accession numbers Z73475 and Z73483). The criteria used when we selected the primer pairs were as follows: (i) a short product (< 600 nucleotides) which would be more likely recovered in samples containing mainly host DNA (Saiki 1990), and (ii) primer sites conserved across *Plasmodium* and *Haemoproteus* but sufficiently different at the 3'-end in the warblers to prevent amplification of their cytochrome *b* gene. We decided to use the primers HAEMF (5'-ATGGTGCCTTTCGATATATGCATG-3') and HAEMR2 (5'-GCATTATCTGGATGTGATAATGGT-3') which we expected to amplify a fragment of 478 nucleotides (excluding the primers). Tests on blood samples from specimens of various passerine genera have shown that the primers are sufficiently different so as to prevent amplification of avian cytochrome *b* genes.

A PCR was performed in volumes of 25 µl which included 25 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM MgCl₂, 1 × PCR buffer (Perkin Elmer), 0.6 µM of each primer and 0.5 units of Taq DNA polymerase. The PCRs including the primers HAEMF and HAEMR2 were run using the following conditions: 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C (35 cycles). Before the cyclic reactions, the samples were incubated at 94 °C for 3 min and after completion at 72 °C for 10 min. To check the success of the reactions, we used 2.5 µl of the final PCRs for running on a 2% agarose gel.

We mainly used samples of DNA extracted from blood for the present study in order to screen opportunistically for the presence of positive PCRs (table 1). However, for two species, the great reed warbler (*Acrocephalus arundinaceus*) and the blue tit (*Parus caeruleus*), we also compared our PCR-based method for determining the presence or absence of *Haemoproteus* and/or *Plasmodium* in individual birds with the traditional method of visually inspecting blood smears under a microscope. Blood samples were obtained from a population of great reed warblers in south Central Sweden (Bensch *et al.* 1998) and from a population of blue tits in south Sweden (Svensson & Nilsson 1995). Blood smears were air-dried, fixed in methanol and later

stained in Giemsa stain. The smears were screened for haematozoa by two of the authors (Ö.Ö. for great reed warblers and M.S. for blue tits). The parasite intensity (parasitaemia) was estimated as the proportion of infected red blood cells. All the samples of great reed warblers ($n=8$) and blue tits ($n=10$) analysed and scored to be parasitized by *Haemoproteus* yielded a single PCR product of the expected length (figure 1). The parasite intensity, which was measured as the percentage of infected red blood cells on the blood smears, varied between 0.04 and 7.9% in these individuals. We found three samples in the great reed warblers which showed positive amplifications from 30 which were previously scored to be parasite free. The blood smears from these individuals were reinspected and proved to be of poor quality showing large proportions of damaged blood cells. None of the ten blue tit samples analysed from individuals, scored to be parasite free, yielded positive PCR products. Hence, this PCR-based protocol seems to be as effective in detecting the prevalence of *Haemoproteus* and *Plasmodium* as traditional inspection of blood smears.

Samples showing positive amplifications were selected for sequencing. We took 15 µl of the remaining PCR product to be precipitated by adding 8 µl of 8 M NH₄Ac and 24 µl of ethanol. Following centrifugation, the air-dried DNA pellet was dissolved in 15 µl of water. The fragments were sequenced directly from both ends with the primers HAEMF and HAEMR2 using dye terminator cyclic sequencing (big dye) and loaded on an ABI PRISMTM 310 (Perkin Elmer).

The sequences were edited and aligned using the program BioEdit (Hall 1999). In order to estimate the phylogenetic relationships between the obtained and published avian *Haemoproteus* and *Plasmodium* haplotypes we used the program MEGA (Kumar *et al.* 1993) and the neighbour-joining method. We used a Kimura two-parameter distance for the DNA sequence data under a gamma distribution ($\alpha=0.24$) estimated in PUZZLE (Strimmer & Von Haeseler 1996) from the present data set, whereas the amino-acid sequence data were analysed simply as the number of differences. The sequences have been deposited at the GenBank International Nucleotide Sequence Database with accession numbers AF254962–AF254978.

The phylogenetic relationships between the 12 passerine birds investigated here and the three bird species for which *Haemoproteus* or *Plasmodium* sequences are published (Escalante *et al.* 1998) were derived from published phylogenies of *Phylloscopus* (Richman & Price 1992) and *Acrocephalus* (Helbig & Seibold 1999) and the relationship of these genera to *Parus*, *Passer*, *Columba* and *Gallus* (Sibley & Ahlquist 1990; Sheldon & Gill 1996).

3. RESULTS

We obtained 17 different mitochondrial lineages. All of the mitochondrial lineages were found in one host species only, except PARUS1, which was detected in both great tits and blue tits. The neighbour-joining method using the amino-acid sequence data identified two tight clusters (figure 2). The separation of the haplotypes into these two major clades was supported by the analysis of the DNA sequence differences which also increased the resolution within these two clusters (figure 3). We interpret the top clusters in figures 2 and 3 as representing different species or mitochondrial lineages of *Haemoproteus*. Morphological examination of the blood smears from individuals with GRW1 and PARUS1 infections tentatively identified them

Table 1. *Host species, site of sampling, number of screened individuals and detected Haemoproetus/Plasmodium haplotypes*

(Samples chosen from individuals showing blood smears positive for *Haemoproetus* or *Plasmodium* are denoted by an asterisk. Only one haplotype was detected per individual, with the exception of one greenish warbler which seemed to carry a mix of GW1 and a *Plasmodium*-like haplotype.)

host species	site of sampling	number tested	detected haplotypes (number of individuals)
great reed warbler <i>Acrocephalus arundinaceus</i>	Sweden	10*	GRW1 (9), GRW2 (1)
	Latvia	10	GRW1 (1), GRW2 (1)
	Germany	19	GRW1 (4)
	Greece	9	GRW2 (1)
	Israel	6	GRW1 (1), GRW4 (1)
	Kenya	10	GRW3 (1), GRW4 (1)
oriental reed warbler <i>Acrocephalus orientalis</i>	Japan	10	ORW1 (1)
basra reed warbler <i>Acrocephalus griseldis</i>	Kenya	10	BRW1 (4)
marsh warbler <i>Acrocephalus palustris</i>	Sweden	14	MW2 (1)
	Kenya	20	MW1 (2)
sedge warbler <i>Acrocephalus schoenobaenus</i>	Sweden	27	SW1 (2)
reed warbler <i>Acrocephalus scirpaceus</i>	Sweden	15	—
	Spain	5	RW1 (1)
willow warbler <i>Phylloscopus trochilus</i>	Sweden	100	WW1 (10), WW2 (8)
large-crowned leaf warbler <i>Phylloscopus occipitalis</i>	India	9	LCLW1 (2)
greenish warbler <i>Phylloscopus trochiloides</i>	India	5	GW1 (3)
Hume's yellow-browed warbler <i>Phylloscopus humei</i>	India	10	HLW1 (2)
blue tit <i>Parus caeruleus</i>	Sweden	4*	PARUS1 (4)
great tit <i>Parus major</i>	Sweden	4*	PARUS1 (3), PARUS2(1)

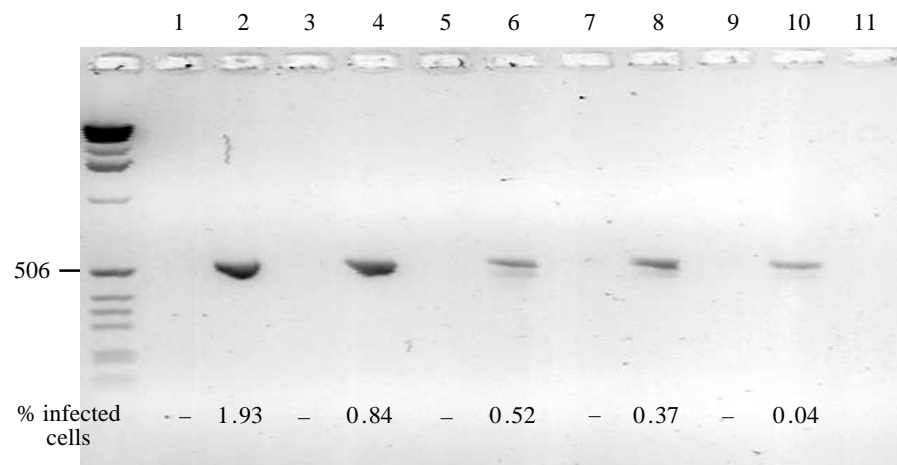


Figure 1. Agarose gel showing the results of a PCR with the primers HAEMF and HAEMR2 on the great reed warbler DNA samples extracted from the blood of birds differing in their levels of *Haemoproetus* infestation (parasitaemia) according to microscopic inspections of blood smears. The template DNAs in the PCRs shown in lanes 1, 3, 5, 7 and 9 were from presumed uninfected birds and in lanes 2, 4, 6, 8 and 10 from birds with 1.93–0.04% of red blood cells infected with *Haemoproetus*. Lane 11 shows a blank reaction. To the left is a DNA size ladder (1 kb DNA ladder Gibco BRL) with the position of the 506 nucleotide fragment indicated.

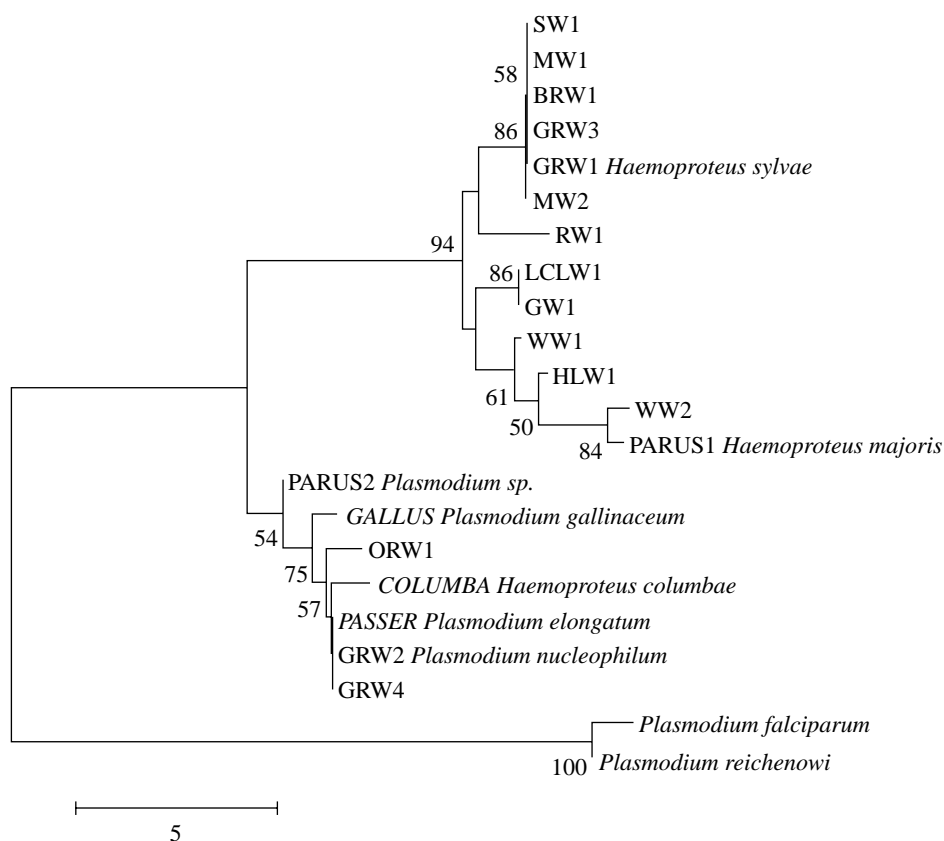


Figure 2. A phylogenetic tree using the amino-acid sequence data of the partial cytochrome *b* gene (478 nucleotides) of *Plasmodium* and *Haemoproteus*. The haplotype abbreviations are as in table 1. The tree was obtained using the neighbour-joining method on the number of amino-acid differences between the sequences. *Plasmodium falciparum* (GenBank number AF069609) and *Plasmodium reichenowi* (GenBank number AF069610) were used as outgroups. Bootstrap values ($> 50\%$) are provided as the percentages over replicates over 500 replications. Scientific names are given for those haplotypes which have been morphologically identified.

as *Haemoproteus sylvae* and *Haemoproteus majoris*, respectively (M. A. Anwar, personal communication). The other cluster instead seemed to contain mainly different *Plasmodium* species, including published sequences of *Plasmodium gallinaceum* (GALLUS) and *Plasmodium elongatum* (PASSER) (Escalante *et al.* 1998) and GRW2 tentatively identified as *Plasmodium nucleophilum* from blood smears (M. A. Anwar, personal communication). However, the almost identical sequence in GRW2 and in COLUMBA (one substitution at position 166) (GenBank number AF069613) obtained from a dove (*Columba livia*) in Venezuela and reported to represent *Haemoproteus columbae* (Escalante *et al.* 1998) is remarkable and questions whether *Haemoproteus* as a genus is monophyletic.

We identified four different parasite mitochondrial haplotypes in the great reed warblers, two within the *Haemoproteus* cluster (GRW1 and GRW3) and two within the *Plasmodium* cluster (GRW2 and GRW4). The DNA sequence divergence between the *Haemoproteus* variants was 0.63% and between the *Plasmodium* variants was 7.2%. Despite the latter (GRW2 and GRW4) being substantially divergent in their DNA sequences (figure 3), they showed identical amino-acid sequences (figure 2). This emphasizes a general feature of the data set as a whole, i.e. that most of the substitutions involve third and first codon positions, which usually do not alter the amino-acid sequences. We found two *Haemoproteus*

variants in the willow warbler with a DNA sequence divergence of 5.2%, one (WW1) clustering with a haplotype detected in a Hume's yellow browed warbler (HLW 1) (*Phylloscopus humei*) (0.84% DNA sequence divergence) and the other (WW2) clustering with the PARUS1 haplotype detected in the two tit species (0.63% DNA sequence divergence).

The constructed phylogenies of the parasites and those of their host species matched poorly within the *Haemoproteus* cluster (figure 3). Most notable was the WW2 haplotype in the willow warblers which clustered with the PARUS1 haplotype encountered in the two tit species. We found a tight cluster of six *Haemoproteus* haplotypes with only slight sequence divergence in four species of *Acrocephalus* warblers. The sequence divergence among these six *Haemoproteus* haplotypes was 0.2–2.1%, whereas the corresponding cytochrome *b* divergence among the four *Acrocephalus* warblers ranged between 9.6 and 11.8% (Helbig & Seibold 1999). For example, one of the haplotypes obtained in the great reed warblers (GRW1) differed from a haplotype obtained in basra reed warblers (*Acrocephalus griseldis*) at only one substitution (0.2%) (BRW1), although these two bird species showed a cytochrome *b* divergence of 9.8% (Helbig & Seibold 1999).

The different *Plasmodium* haplotypes appeared to be more distantly related than the *Haemoproteus* haplotypes

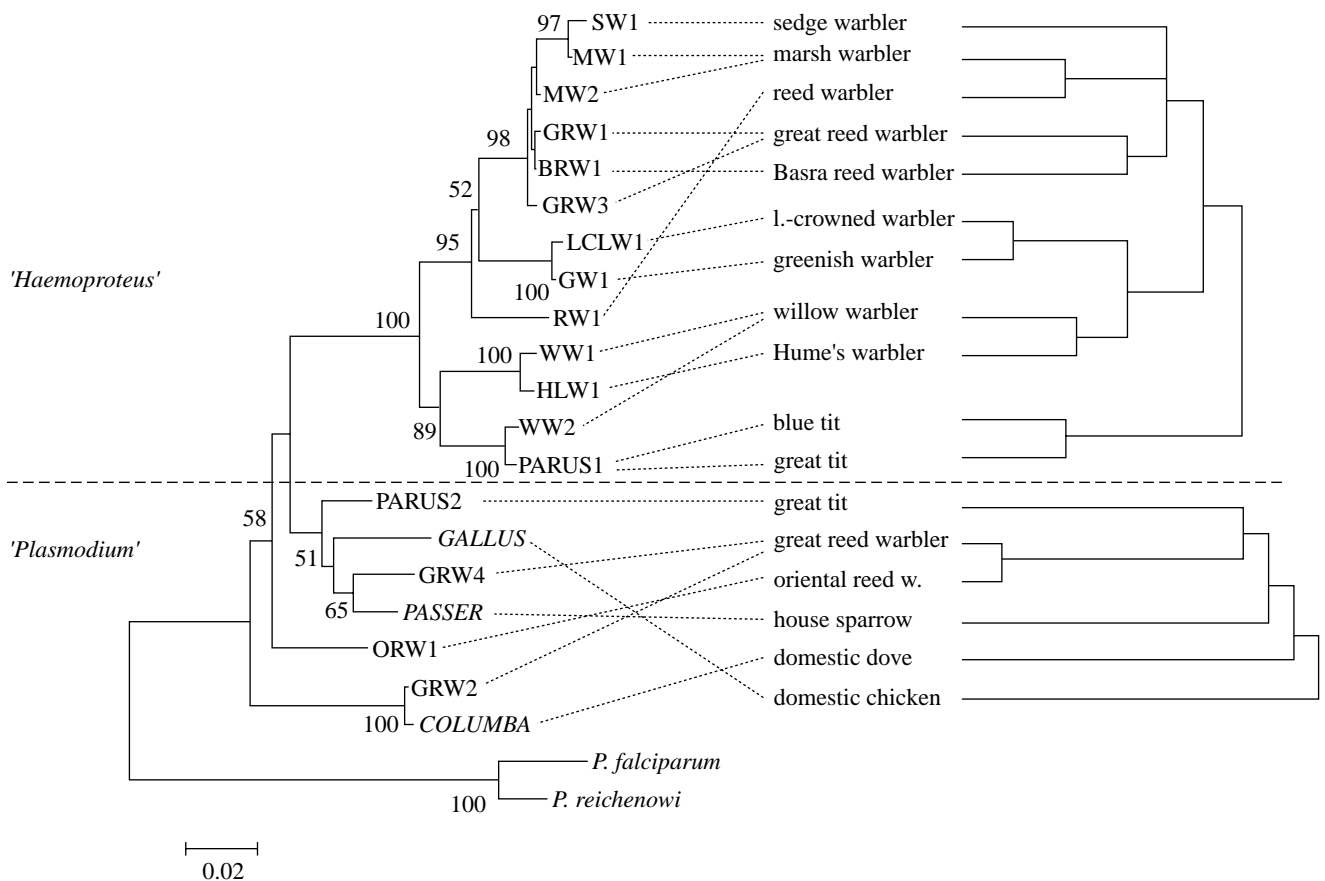


Figure 3. A phylogenetic tree using the DNA sequence data of the partial cytochrome *b* gene (478 nucleotides) of *Plasmodium* and *Haemoproteus* (left) and phylogenetic trees of the bird species for which the *Haemoproteus* and *Plasmodium* sequences were obtained (right). The dotted lines connect the host species with the parasite haplotypes encountered. The haplotype abbreviations and scientific names are as in table 1. The *Plasmodium* and *Haemoproteus* tree was obtained using the neighbour-joining method with the Kimura two-parameter distance and a gamma distribution ($\alpha=0.24$). *Plasmodium falciparum* (GenBank number AF069609) and *Plasmodium reichenowi* (GenBank number AF069610) were used as outgroups. Bootstrap values ($> 50\%$) are provided as the percentages over replicates over 500 replications. The phylogenetic relationships between the bird host species studied were constructed from published phylogenies based on mtDNA sequences (Richman & Price 1992; Helbig & Seibold 1999) and DNA–DNA hybridization data (Sibley & Ahlquist 1990; Richman & Price 1992; Sheldon & Gill 1996).

and possibly matched the phylogenetic relationships of their host species even less well (figure 3). For example, the GRW4 haplotype from the great reed warblers clustered with the PASSER haplotype from house sparrows (*Passer domesticus*) and the GALLUS haplotype from domestic chickens (*Gallus gallus*), respectively (Escalante *et al.* 1998).

4. DISCUSSION

The phylogenetic tree constructed from the parasites' DNA showed a poor match with the phylogenetic tree of their host bird species. This result suggests that host shifts have occurred several times during the evolution of these species, which emphasizes the risk of avian host species being infected by novel parasites. For example, we found the sequence divergence among six *Haemoproteus* haplotypes detected in four species of *Acrocephalus* to be only 0.2–2.1%, whereas the corresponding cytochrome *b* divergence among the birds was *ca.* 10% (Helbig & Seibold 1999). Hence, in order for the *Haemoproteus* lineages to have evolved in parallel with their host species, a rate difference in molecular evolution of one order of magnitude is

required between the parasite and host cytochrome *b* genes. That the two tit species and the willow warbler carry parasites differing by only 0.6% sequence divergence suggests that *Haemoproteus* not only shifts between species within the same genus but also between species in different families. The divergence time between *Parus* and *Phylloscopus* is *ca.* 30 million years (Myr) using DNA–DNA hybridization data (Sibley & Ahlquist 1990), assuming a split between chickens and ostriches 80–90 Myr ago (Härlid *et al.* 1997).

A less than perfect match between parasite and host phylogenies can potentially have other explanations than host switching (Page & Charleston 1998). For example, if multiple lineages of a parasite exist within a host (resulting from duplication) and the host is split into two or more lineages, some parasite lineages may have been missing in some of the host lineages from the beginning ('missing the boat') or may have become extinct at a later stage of divergence. Such sorting events may result in incongruent phylogenies between parasites and hosts. We found that many of the *Haemoproteus* lineages differed by $\leq 1\%$ sequence divergence suggesting that they have diverged recently. In contrast, these relatively similar

parasite lineages were encountered in different bird species which, according to their mtDNA distances, have been separated for several millions of years. Sorting is unlikely to produce such a pattern, unless the birds have a tenfold higher rate of molecular evolution relative to their parasites.

Haemoproteus are believed to be host specific or at least nearly so (Atkinson & Van Riper 1991). However, the current taxonomy is mainly based on host species and the morphology of the parasites found in circulating red blood cells (Atkinson & Van Riper 1991). Because *Haemoproteus* are only represented by gametocytes in the blood, transindividual inoculations are not possible and the theory of host specificity cannot be tested experimentally. Our results support the hypothesis that most species presently have their own *Haemoproteus* parasite (Atkinson & Van Riper 1991); however, in many cases these differ by less than 1% sequence divergence. In addition, some species, such as the great reed warbler, the willow warbler and the marsh warbler, are the target of more than one *Haemoproteus* parasite. The number of different parasite lineages per host is likely to be much larger than detected in this study because of the low sampling effort in most species.

To what extent do the different mitochondrial lineages represent different parasite species? Presently, we do not know enough about the rate of mitochondrial evolution within this group of parasites to translate the sequence differences directly into taxonomic distinctions. The haplotype GRW1 was classified as *H. sylvae*, whereas that from PARUS1 was classified as *H. majoris* (the sequence divergence between these taxa is 6.6%). The 5.2% difference detected between the two willow warbler variants corresponds to the average distance between sister species of birds (Johns & Avise 1998). This difference is also slightly larger than the genetic distance between the human parasite *Plasmodium falciparum* and chimpanzee parasite *Plasmodium reichenowi*, which probably diverged ≥ 4 Myr ago (Escalante *et al.* 1998).

The genera *Haemoproteus* and *Plasmodium* are distinguished by the latter not only having gametocytes but also asexually reproducing merozoites in their red blood cells and by the number and spacing of microtubules in their sporozoites (Carreno *et al.* 1997). However, evaluating the two latter characters is not possible from blood smears. Hence, *Plasmodium* and *Haemoproteus* are mainly separated on the basis of different life cycles, which may not reflect phylogeny. This was recently emphasized by Escalante *et al.* (1998) who showed that *H. columbae* clustered tightly with two avian *Plasmodium* species and that *Plasmodium* as a whole was polyphyletic. In the present study, we found that the *Plasmodium* and *Haemoproteus* mtDNA sorted mainly into two different clades except for the placement of *H. columbae* among the avian *Plasmodium* variants. The published sequence of *H. columbae* (Escalante *et al.* 1998) is almost identical to the sequence obtained in this study from three great reed warblers classified as being infected by *P. nucleophilum*. Further studies are needed in order to establish whether two such different parasites as *H. columbae* and *P. nucleophilum* indeed have almost identical cytochrome *b* nucleotide sequences.

Haematozoans have frequently been used as model parasites in population studies of vertebrates because

their prevalence is relatively easy to quantify by microscopic examination of blood smears obtained from free-ranging animals. One limitation of the visual method when testing the effects of the parasites on the fitness of their host is that different strains of the parasites might appear identical in morphology, but have different pathological effects on infected host individuals. We found that this PCR-based method is equally good as microscopy in scoring for parasite prevalence in great reed warblers and blue tits, corroborating two other PCR-based studies targeting 18S rRNA genes which amplify an intron of different length in birds–reptiles and *Plasmodium* (Feldman *et al.* 1995; Perkins *et al.* 1998). In addition, PCR-based methods allow the separation of closely related parasite strains. In the years to come, we expect that molecular studies of avian haematozoa will reveal many new and exciting aspects of host–parasite evolution, including the degree of host specificity, geographical variation and species-specific pathology. An interesting possibility is that the parasite species composition could be used to discriminate between populations during migration and on wintering grounds, as suggested for willow warblers (Rintamäki *et al.* 1998) and such an approach would of course be more powerful with information about the parasites mtDNA. A PCR-based technique would also allow the screening of possible vectors (Mathiopoulos *et al.* 1993) and, hence, conclusively determine the complete life history of these organisms which are currently mainly known as small granulated structures in avian erythrocytes.

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