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LINKAGE BETWEEN NUCLEAR AND MITOCHONDRIAL DNA SEQUENCES IN AVIAN MALARIA PARASITES: MULTIPLE CASES OF CRYPTIC SPECIATION?

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Abstract.—Analyses of mitochondrial cytochrome *b* diversity among avian blood parasites of the genera *Haemoproteus* and *Plasmodium* suggest that there might be as many lineages of parasites as there are species of birds. This is in sharp contrast to the approximately 175 parasite species described by traditional methods based on morphology using light microscopy. Until now it has not been clear to what extent parasite mitochondrial DNA lineage diversity reflects intra- or interspecific variation. We have sequenced part of a fast-evolving nuclear gene, dihydrofolate reductase–thymidylate synthase (DHFR-TS), and demonstrate that most of the parasite mitochondrial DNA lineages are associated with unique gene copies at this locus. Although these parasite lineages sometimes coexist in the same host individual, they apparently do not recombine and could therefore be considered as functionally distinct evolutionary entities, with independent evolutionary potential. Studies examining parasite virulence and host immune systems must consider this remarkable diversity of avian malaria parasites.

Key words.—Cytochrome *b*, DHFR-TS, *Haemoproteus*, *Plasmodium*, reproductive isolation.

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Avian malaria parasites of the genera *Haemoproteus* and *Plasmodium* multiply as haploid clones in avian hosts and undergo sexual reproduction in vectors, which for *Haemoproteus* ssp. are primarily biting midges *Culicoides* (Desser and Bennett 1993) and for avian *Plasmodium* ssp. *Culex* mosquitoes (van Riper et al. 1993). With traditional methods using light microscopy to evaluate parasite morphology, researchers have recognized approximately 175 species of avian malaria parasites of the genera *Haemoproteus* and *Plasmodium* (Valkiunas 1997). The delineation of species in this group of organisms is a contentious issue, and, historically, some taxonomists have used avian host species as an additional criterion to identify parasite species (Garnham 1966). With this approach, morphologically identical parasites were given different names if observed in different species of avian hosts. However, the ''one host–one parasite'' argument of defining species assumes that parasites do not shift or share host species, an assumption that has been rejected by recent molecular studies (Waldenström et al. 2002).

In the present study, we are not attempting to define a species concept for avian malaria parasites. Rather, we are focusing on one important aspect of any species concept, namely that nonrecombining lineages are independent evolutionary entities (Hey et al. 2003). The presence of nonrecombining parasite lineages within the same host species and geographical area indicates that such lineages are good species according to the biological species concept, and this is the species concept we have in mind in the present paper. Given time, however, such isolated lineages have the potential to evolve into distinct species as defined by other species criteria, for example, based on morphology, vector specificity, or host distribution.

Recently developed methods based on polymerase chain reaction (PCR; Jarvi et al. 2002; Richard et al. 2002; Fallon et al. 2003b) are generating sequence data that provide much more detailed information of malaria parasite lineages than could be retrieved from morphology alone, allowing for the reconstruction of their evolutionary history (Perkins and Schall 2002) and identification of cryptic species (Perkins 2000). Estimates from observed cytochrome *b* (cyt *b*) sequence diversity of avian malaria parasites (Ricklefs and Fallon 2002; Waldenström et al. 2002) suggest that there might be as many species of parasites as there are species of bird hosts. These findings contribute to, at least partly, resurrecting the early taxomic idea of one host–one parasite. For example, Ricklefs and Fallon (2002) found 68 distinct avian malaria lineages in a sample of 79 species of avian hosts and Waldenström et al. (2002) found 18 parasite lineages in nine species of birds. Hence, rather than a few hundred parasite species as suggested by the morphologically based taxonomy, the true number of species might approach 10,000 to match the number of bird species. This estimate is, however, solely based on parasite mitochondrial diversity, and gene genealogies do not necessarily correspond to species phylogenies (Hudson and Coyne 2002). Thus, at present it is unknown at what level parasite cyt *b* sequence divergence corresponds to the delineation of parasite species. Intraspecific cyt *b* divergence reaches 4% in some mammal species (Good and Sullivan 2001) but is only 0.2% in the human parasite *P. falciparum* (Joy et al. 2003). However, closely related avian malaria mitochondrial DNA (mtDNA) lineages are often restrictively distributed among host species: parasite cyt *b* lineages with less than 1% sequence divergence have been observed to be confined to separate host species (Bensch et al. 2000; Waldenström et al. 2002; Ricklefs et al. 2004). In such cases the lineages are presumable reproductively isolated; however, it is not clear for how long they have been isolated and to what extent they share nuclear genetic variation. A direct way of testing these hypotheses is to compare sequences for mitochondrial and nuclear genes obtained from the same parasite lineages.

For this purpose we developed conserved primers amplifying a partial segment of the DHFR-TS gene of avian malaria parasites. This gene encodes a bifunctional enzyme composed of dihydrofolate reductase (DHFR) and thymidylate synthase (TS; Garrett et al. 1984). The DHFR-TS enzyme is the target

TABLE 1. Cytochrome *b* lineage, tentative parasite species names, and host species for the samples analyzed for the DHFR-TS locus.

Cytochrome *b* lineage	Parasite species	Host species	Locations of samples positive at the DHFR-TS locus	Detected DHFR-TS lineages (number of individuals sequenced/tested)	GenBank accession no.
GRW1	*Haemoproteus payevskyi*	*Acrocephalus arundinaceus*	Sweden[1]	A (9/12)	AY560361
GRW8	*Haemoproteus payevskyi*	*Acrocephalus arundinaceus*	Sweden[1]	A (1/2)	AY560362
BRW1	*Haemoproteus payevskyi*	*Acrocephalus griseldis*	Kenya[2]	B (2/3)	AY560363
GRW5	*Haemoproteus payevskyi*	*Acrocephalus arundinaceus*	Sweden[1]	C (1/1)	AY560364
SYAT2	*Haemoproteus belopolskyi*	*Sylvia atricapilla*	Spain[3]	D (4/18)	AY560365
SYAT16	*Haemoproteus belopolskyi*	*Sylvia atricapilla*	Spain[4]	E (1/1)	AY560366
SYAT7	*Haemoproteus belopolskyi*	*Sylvia atricapilla*	Sweden[5]	F (1/1)	AY560367
SYAT1	*Haemoproteus belopolskyi*	*Sylvia atricapilla*	Spain[6]	G (4/22)	AY560368
PARUS1	*Haemoproteus majoris*	*Parus caruleus*	Sweden[5]	H (16/16)	AY560369
SYAT3	*Haemoproteus* sp.	*Sylvia atricapilla*	Spain[6]	I (1/1)	AY560370
WW2	*Haemoproteus majoris*	*Phylloscopus trochilus*		− (−/8)	
WW1	*Haemoproteus* sp.	*Phylloscopus trochilus*	Sweden[5]	J (2/8)	AY560371
SGS1	*Plasmodium* sp.	*Acrocephalus arundinaceus*		− (−/4)	
SGS1	*Plasmodium* sp.	*Sylvia atricapilla*	Spain[7]	K (1/2)	AY560372
GRW2	*Plasmodium* sp.	*Acrocephalus arundinaceus*	Sweden[1]	L (1/1)	AY560373

[1] Lake Kvismaren (central Sweden).
[2] Tsavo National Park.
[3] One bird from Alava (north Spain), two from Guadarrama (central Spain), and one from Ojen (Gibraltar area).
[4] Alava (northern Spain).
[5] Lake Krankesjön (south Sweden).
[6] Ojen (Gibraltar area).
[7] Guadarrama (central Spain).

for the antimalarial drug pyrimethamine (Zhang and Rathod 2002) and alleles for drug resistance have been recorded both for *P. falciparum* and *P. vivax* at this specific locus (Sirawaraporn et al. 1997; de Pécoulas et al. 1998). The DHFR-TS gene apparently has no introns (Peterson 2001), which makes it particularly useful for phylogenetic reconstructions. Apart from the human malaria species, the gene has been sequenced in several species of rodent malaria (Cowman and Lew 1989) and in the avian malaria species *P. gallinaceum* (Peterson 2001). This made it possible to identify conserved regions for location of primers.

We decided to focus on two groups of morphologically identified *Haemoproteus*, both containing many similar cyt *b* parasite lineages (Waldenström et al. 2002); one group tentatively belonging to *H. payevskyi* obtained from two species of *Acrocephalus* warblers and one group of *H. belopolskyi* from blackcaps, *Sylvia atricapilla*. These bird species were chosen based on previous studies (Waldenström et al. 2002; J. Pérez-Tris, unpubl. data) as these hosts have been shown to harbor several closely related cyt *b* lineages of parasites. If similar cyt *b* sequences represent different species of parasites, we predict that they should be associated with different, and unique, sequences at the DHFR-TS locus. However, if similar cyt *b* sequences represent a within-species polymorphism in mtDNA, they should be associated with identical DHFR-TS alleles, or if different alleles are detected, this variation should be shared across the mtDNA lineages.

MATERIALS AND METHODS

We used samples for which we previously had amplified and sequenced avian malaria cyt *b* sequences. We focused on *Haemoproteus* lineages in great reed warblers, *A. arundinaceus* (collected in Sweden); basra reed warblers, *A. griseldis* (Kenya); and blackcaps, *S. atricapilla* (Sweden and various locations in Spain, see Table 1 for details). To increase the sample of reference sequences, we also opportunistically tried to amplify *Plasmodium* lineages from the above mentioned host species, and *Haemoproteus* lineages from blue tits, *Parus caruleus* (Sweden), and willow warblers, *Phylloscopus trochilus* (Sweden). Blood sampling, DNA extraction, and amplification and sequencing of partial cyt *b* sequences have been described elsewhere (Bensch et al. 2000; Waldenström et al. 2002). We constructed the primers DHFR2F (5′-AAAAAYGTRAGRATATGGGAAGC-3′) and DHFR1R2 (5′-ATATGACAAGGTGGTAATGCCA-3′) to amplify 285 bp (including primers) of the TS domain of the DHFR-TS locus of *Haemoproteus* and *Plasmodium*. PCR reactions were performed in 25-μl total volumes including 25 ng of total genomic DNA, 0.125 mM of each nucleotide, 3.0 mM $MgCl_2$, 1X PCR buffer (Applied Biosystems, Foster City, CA), 0.6 μM of each primer, and 0.5 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). We used a touch-down temperature profile, starting with 12 cycles with a successive reduction of the annealing temperature by 0.7°C from 60°C to 51°C, followed by 28 cycles with a constant annealing temperature (30 sec at 94°C, 30 sec at 51°C, 45 sec at 72°C). Amplified fragments were sequenced directly from both ends with the original primers and using BigDye chemistry (ver. 1.1, Applied Biosystems) and loaded on an ABI Prism 3100 (Applied Biosystems). A total length of 238 bp was sequenced from each of 44 infected individuals.

Sequences were edited and aligned using the software BioEdit (Hall 1999). Phylogenetic trees were estimated with the maximum likelihood quartet puzzling method using the software TREE-PUZZLE 5.0 with eight gamma-distributed rate categories, an HKY model of substitution (Hasegawa et al. 1985), and parameters estimated from the datasets (using the approximate method and a neighbor-joining tree; and Strimmer and von Haeseler 1996). Trees were visualized using TREEVIEW (Page 1996). The congruence between the

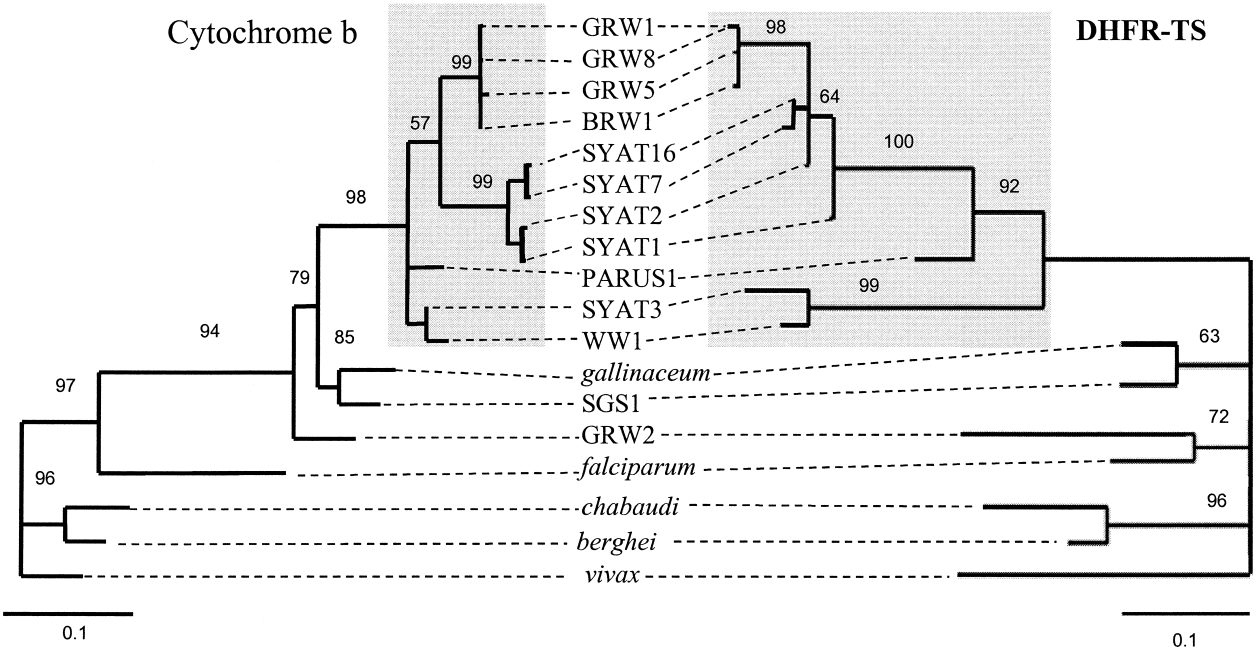

FIG. 1. Genetic relationships between avian malarial parasites constructed from nucleotide sequences of the mitochondrial cytochrome *b* gene (left) and the nuclear DHFR-TS gene (right). The trees were constructed using maximum likelihood and using *Plasmodium vivax* as the root, though other nonavian parasites were also included (*P. berghei*, *P. chabaudi* and *P. falciparum*). Shaded areas denote *Haemoproteus* parasites. Stippled lines connect cytochrome *b* lineage names or species names (italics) with the positions in the two gene trees. Numbers on the branches refer to bootstrap values and branches with a support < 50% have been collapsed.

fully resolved mtDNA and nuclear gene trees was estimated by comparing quartets using the software COMPONENT version 2.00a (Page 1993) and was compared with the distribution of mismatching quartets between 1000 randomly generated trees.

RESULTS

We tested 1–22 individuals infected with previously identified cyt *b* lineages with the primers for the DHFR-TS gene. Generally, the success rate for the DHFR-TS experiment was less than 50% for many of the lineages (Table 1) and thus much lower than for the cyt *b* gene. For example, we failed completely to amplify parasite DHFR-TS from willow warblers infected with the WW2 cyt *b* lineage. From blue tits infected with PARUS1 we successfully amplified the nuclear marker from all 16 samples, which, most likely, was a result of selecting blood samples from individuals with high levels of parasitemia (1.2–6.2% infected erythrocytes). The failures consisted of negative PCR reactions, poor amplifications unsuitable for sequencing, or coamplifications of nonspecific DNA fragments. The lower copy number of nuclear compared to mitochondrial genes probably causes the lower success rate for amplifying the DHFR-TS than cyt *b* gene.

In the total dataset, we found 12 (A–L) different sequences at the DHFR-TS locus. These were strongly and consistently associated with the previously identified cyt *b* lineages (Table 1). Moreover, we obtained almost identical phylogenetic trees of the parasites for the DHFR-TS and the cyt *b* genes (Fig. 1). With 18 terminal taxa, there are 3060 possible quartets, and with the program COMPONENT we found 399 of these to be different. This is a much smaller value than the estimates from randomly compared trees ($P < 0.001$), which on average had 2037 different quartets (minimum 1459). Hence, the two genes appear to evolve in parallel, consistent with a hypothesis of no or little exchange of nuclear genetic material across mitochondrial lineages. Even some of the very similar cyt *b* lineages (< 0.5% sequence divergence) were associated with different sequences at the DHFR-TS locus (e.g., GRW1/GRW5/BRW1 and SYAT1/SYAT2). In the samples analyzed for SYAT1 ($n = 4$ had all G) and SYAT2 ($n = 4$ had all D), the pattern of lineage-specific nuclear sequences were consistent (Table 1). The probability of obtaining this association by chance in a recombining population (assuming the same frequency of the nuclear copies D vs. G) is $P = 0.008$ (2×0.5^8). Similarly, the probability of obtaining unique nuclear sequences for the GRW1 ($n = 9$) and GRW5 ($n = 1$) is $P = 0.039$ (assuming an allele frequencies of A at 0.9 and of C at 0.1). The only case of identical DHFR-TS sequences found across mitochondrial lineages was between GRW1 and GRW8. These two lineages differ by one substitution in the analyzed cyt *b* sequence (0.2% divergence), and might represent a case of intraspecific mitochondrial polymorphism or two species with identical DHFR-TS genes.

DISCUSSION

Cryptic Speciation in Avian Malaria

The strong association of genetic variation between mitochondrial and nuclear loci demonstrates that the different parasite cyt *b* lineages indeed represent reproductively isolated entities. Within the morphologically identified species *H. payevskyi* we found three lineages that do not appear to

recombine. The lack of recombination between BRW1 and GRW1/GRW5/GRW8 cannot be explained by geographic isolation because ranges of the two hosts (basra reed warblers and great reed warblers) overlap during winter in Africa, where the transmission seems to take place (Waldenström et al. 2002). Within the morphologically identified species *H. belopolskyi* we found no less than four nonrecombining lineages coexisting in the same study population. Because the parasite mtDNA lineages represent independent evolutionary entities, each of them could qualify to be considered as a distinct biological species.

The causes of isolation between lineages and their temporal stability are not yet known. Our data do not preclude that these species can interbreed if the ecological situation is changed, as is the case for many ''good'' species in other group of organisms (Seehausen et al. 1997). For example, some of these parasites might have the potential to mix, would they come into contact in a single vector individual where sexual reproduction takes place (Atkinson and van Riper 1991). These opportunities for recombination seem to be common, particularly for the mitochondrial lineages analyzed in this study. In the blackcap, we have found five individuals simultaneously infected with SYAT1 and SYAT2, and one individual simultaneously infected with SYAT1 and SYAT16 (J. Pérez-Tris and S. Bensch, unpubl. data). Despite these obvious possibilities for mixing, recombination between these lineages appears rare or absent.

Important isolating mechanisms that presently are keeping the parasite lineages apart should be geographic distribution of specific vectors and hosts and temperature requirements for sexual reproduction and maturation in vectors. Given the sympatric occurrence of different parasite lineages, not only within the same geographical region but also coinfecting the same host, other reproductive isolating mechanisms must be involved. For example, although different parasite species may occur simultaneously in the same avian host individual and are potentially transmitted by the same vectors (Valkiunas et al. 2002), interspecific interference between parasites during fertilization may prevent their hybridization (Paul et al. 2002).

Implications of High Parasite Diversity

If most of the mitochondrial lineages correspond to different biological species, as our data suggest, the number of avian malaria parasite species (*Plasmodium* spp. and *Haemoproteus* spp.) might approach 10,000, which is two orders of magnitude larger than based on parasite morphology (Valkiunas 1997). It is interesting to note that these recent molecular findings actually corroborate the estimate of species diversity suggested from the old one host–one parasite hypothesis (Garnham 1966). This estimate was, however, right for the wrong reason; the recent molecular analyses have clearly rejected the one host–one parasite hypothesis as a substantial fraction of lineages appear to infect multiple host species (Ricklefs and Fallon 2002; Waldenström et al. 2002). Though many of these suggested species are cryptic in morphology and presumably recently derived, these parasite lineages are evolutionary independent, meaning that the different parasites are free to evolve novel adaptations to their particular environment. For example, the different parasite lineages are expected to evolve adaptations to escape host and vector immune responses and to adjust their developmental temperature tolerance in vectors when spread to areas with different climates.

The high diversity of reproductively isolated parasite lineages infecting the same host species suggests that single host individuals frequently might contract simultaneous and multiple infections. In great reed warblers and blackcaps, we have found such multiple infections, including two or more parasite lineages, in 2.8% ($n = 321$) and 24% ($n = 95$) of infected individuals (S. Bensch, D. Hasselquist, and J. Waldenström, unpubl. data). It has been demonstrated theoretically that within-host competition between genetically distinct parasite lineages is among the most important factors selecting for increased virulence (Frank 1996). Hence, host species exposed to many parasite lineages are not only facing an increased risk of contracting disease, but the high parasite diversity and associated occurrence of multiple infections might also increase the virulence of the parasites.

The large number of malaria parasite lineages, perhaps globally approaching the order of 10,000, and the fact that several lineages may coexist in one host species (23 *Haemoproteus* lineages detected in blackcaps; J. Pérez-Tris, unpubl. data) and be shared with other hosts (Waldenström et al. 2002; Fallon et al. 2003a), sets a scenario of magnificent complexity in terms of parasite-host evolution (Ricklefs et al. 2004). Avian malaria parasite lineages might change in frequency both in time and space (Bensch and Åkesson 2003) and compete over hosts (Paul et al. 2002; Fallon et al. 2003a). Such complex dynamics should promote maintenance of genetic variation of the host immune system (Hedrick 2002), an important prerequisite behind the good-genes model of sexual selection (Hamilton and Zuk 1982; Andersson 1994).

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