International Journal for Parasitology xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Host associations and evolutionary relationships of avian blood parasites from West Africa

Jon S. Beadell [a,*,1], Rita Covas [b,2], Christina Gebhard [c], Farah Ishtiaq [a], Martim Melo [b], Brian K. Schmidt [c], Susan L. Perkins [d], Gary R. Graves [c], Robert C. Fleischer [a]

[a] Center for Conservation and Evolutionary Genetics, National Zoological Park, Smithsonian Institution, 3001 Connecticut Avenue, Washington, DC 20008, USA
[b] CEFE-CNRS, 1919 Route de Mende, F-34293 Montpellier, France
[c] Department of Vertebrate Zoology, MRC-116, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, Washington, DC 20013-7012, USA
[d] Sackler Institute for Comparative Genomics and Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

ARTICLE INFO

Article history:
Received 17 April 2008
Received in revised form 13 June 2008
Accepted 16 June 2008
Available online xxxx

Keywords:
DHFR-TS
Haemoproteus
Host-specificity
Mitochondrial lineage
Plasmodium
Transferase

ABSTRACT

The host specificity of blood parasites recovered from a survey of 527 birds in Cameroon and Gabon was examined at several levels within an evolutionary framework. Unique mitochondrial lineages of *Haemoproteus* were recovered from an average of 1.3 host species (maximum = 3) and 1.2 host families (maximum = 3) while lineages of *Plasmodium* were recovered from an average of 2.5 species (maximum = 27) and 1.6 families (maximum = 9). Averaged within genera, lineages of both *Plasmodium* and *Haemoproteus* were constrained in their host distribution relative to random expectations. However, while several individual lineages within both genera exhibited significant host constraint, host breadth varied widely among related lineages, particularly within the genus *Plasmodium*. Several lineages of *Plasmodium* exhibited extreme generalist host–parasitism strategies while other lineages appeared to have been constrained to certain host families over recent evolutionary history. Sequence data from two nuclear genes recovered from a limited sample of *Plasmodium* parasites indicated that, at the resolution of this study, inferences regarding host breadth were unlikely to be grossly affected by the use of parasite mitochondrial lineages as a proxy for biological species. The use of divergent host–parasitism strategies among closely related parasite lineages suggests that host range is a relatively labile character. Since host specificity may also influence parasite virulence, these results argue for considering the impact of haematozoa on avian hosts on a lineage-specific basis.

1. Introduction

Host specificity is a key factor influencing the virulence of parasites (Garamszegi, 2006), their geographical ranges and the extent to which parasites may emerge into novel hosts (Taylor et al., 2001; Woolhouse and Gowtage-Sequeria, 2005; Hellgren et al., 2007a,b). Host–parasite associations presumably reflect the physiological and immunological constraints imposed by hosts, as well as the ecological factors, such as the distribution and abundance of hosts, parasites and vectors, which limit opportunities for transmission of parasites between different hosts. While host specialization may limit the availability of resources and increase the risk of extinction, it may also allow for increased contact among individuals of a parasite species restricted to a narrow host range. This, in turn, could increase opportunities for out-crossing, leading to increased genetic diversity and possibly the evolutionary flexibility required to colonize new hosts (Combes and Theron, 2000). On the other hand, parasites with broad host distributions are generally thought to have low fitness in any one host, but may achieve higher abundance and face reduced extinction risk relative to specialists. Given these trade-offs, the evolutionary path followed by a parasite species likely represents a fine balance between the selective pressures favoring either specialist or generalist strategies (Woolhouse et al., 2001). Small perturbations to these pressures over time or across space could lead to reversals in host–parasitism strategies and variability in strategies even amongst closely related parasites.

Avian haematozoa present an interesting system for studying the host–parasitism strategies (specialization or promiscuity) of closely related parasites due to their high diversity and the diverse host fauna that is potentially available to a parasite in any particular geographical location. Avian malaria (family Plasmodidae) and related haematozoan parasites in the families Haemoproteidae and Leucocytozoidae have been detected on every continent

* Corresponding author. Tel.: +1 203 432 3886; fax: +1 203 432 7394.
E-mail address: jon.beadell@yale.edu (J.S. Beadell).
[1] Present address: Department of Ecology and Evolutionary Biology, Yale University, ESC158b, 21 Sachem Street, New Haven, CT 06520-8106, USA.
[2] Present address: CIBIO, University of Porto, Campus Agrario de Vairao, R. Monte-Crasto, 4485-661 Vairao, Portugal; Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, West Mains Road, EH9 3JT Edinburgh, UK.

0020-7519/$ - see front matter © 2008 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2008.06.005

except Antarctica. Excluding a few host taxa restricted to extreme arctic environments (Bennett et al., 1992) and perhaps several remote island taxa (Steadman et al., 1990; Beadell et al., 2007), the vast majority of bird species are hosts to avian haematozoa. Recent application of sensitive molecular techniques to the detection of haematozoa have revealed an extremely broad diversity of parasite lineages (Bensch et al., 2000; Ricklefs and Fallon, 2002; Beadell et al., 2004; Durrant et al., 2006; Ishtiaq et al., 2007; Hellgren et al., 2007a), calling into question previous morphological species limits (Beadell et al., 2006; Hellgren et al., 2007b) and raising the possibility that haematozoan species diversity is on the order of avian species diversity (Bensch et al., 2004) or even higher. Supporting this hypothesis, single host species have been shown to harbor between five and 34 distinct parasite mitochondrial lineages (Fallon et al., 2006; Ishtiaq et al., 2006; Kimura et al., 2006; Bensch et al., 2007; Durrant et al., 2007; Wood et al., 2007), and evidence from corresponding nuclear sequence data suggests that at least some mitochondrial parasite lineages may represent reproductively-isolated units (Bensch et al., 2000; Hellgren et al., 2007a).

Large-scale regional surveys have suggested that avian blood parasites exhibit a wide array of host–parasitism strategies, measured both among parasite genera (typically *Haemoproteus* and *Plasmodium*) and within genera (Beadell et al., 2004; Ricklefs et al., 2004; Fallon et al., 2005; Krizanauskiene et al., 2006). Historically, *Haemoproteus* spp. was considered to be more host-specific than *Plasmodium* spp., possibly owing to greater host fidelity among its vectors (hippoboscid and ceratopogonid flies) relative to mosquitoes. Average host-breadth indices developed for parasites from an insular system (Antilles) provided some evidence for this difference, but also demonstrated that individual lineages of both *Haemoproteus* and *Plasmodium* can infect more than 20 species (Fallon et al., 2005). Similar studies in Australia, North America and Europe have confirmed this general trend, reporting the recovery of individual lineages of both *Haemoproteus* and *Plasmodium* from multiple host species and host families (Beadell et al., 2004; Ricklefs et al., 2004; Krizanauskiene et al., 2006). Because of the extreme diversity of avian haematozoa, however, these studies combined have characterized only a small proportion of the parasite lineages likely to exist.

In order to further explore the variability of host–parasite associations within and among genera of avian haematozoa, we report here the host range and evolutionary relationships of parasites recovered from a relatively restricted geographical region of West Africa. A previous study employing similar methods on a largely distinct group of hosts (Beadell et al., 2004) suggested that *Plasmodium* spp. exhibits very weak host specificity relative to *Haemoproteus*, however specificity was examined only at the level of host family and the results may have been biased due to sparse sampling within the genus *Plasmodium*. Here, we quantify the evolutionary scale on which host–parasite associations have been generated and maintained using a more robust sample of *Haemoproteus* and *Plasmodium* lineages. In addition, we incorporate data from three independent genetic markers in order to help circumscribe the limits of reproductive isolation among parasites, thereby providing a basis for judging the validity of using parasite mitochondrial lineages as a taxonomic unit for quantifying host associations.

2. Materials and methods

2.1. Sample collection, parasite detection and identification

Avian tissue samples were collected by B. Schmidt and C. Gebhard (National Museum of Natural History, Washington DC, USA) from 396 individuals in the Gamba Complex, Gabon during the period February 2002 to April 2003 (Angehr et al., 2006). Blood was collected from an additional 131 individuals mist-netted in Cameroon (Mann's Spring, Buéa and Limbe) and Gabon (Cap Esterias and Kango) by R. Covas and M. Melo (University of Edinburgh, UK) at various seasons between 2002 and 2004 (Fig. 1). Sampling of birds followed the Ornithological Council's Guidelines to the Use of Wild Birds in Research (Gaunt et al., 1997). We extracted DNA from these samples using DNeasy kits (Qiagen) following the manufacturer's protocol. Parasite screening and identification of lineages was performed using methods described previously (Beadell et al., 2004; Beadell and Fleischer, 2005). Briefly, we screened for haematozoan parasites using primer sets F2/R2, 850F/1024R and 213F/372R in a 25 μl PCR. The latter primer set allowed for differentiation of parasites in the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* via restriction fragment length polymorphism (Beadell and Fleischer, 2005). From samples in which we detected an infection, we amplified and sequenced a 533 bp fragment of the cyt *b* gene using primers 3760F/4292rw2. In cases where a low quantity of DNA or degraded template prevented the amplification of this fragment, we sequenced smaller fragments using primers Fifi/4292rw2 (351 bp; Ishtiaq et al., 2006) or F2/4292rw2 (256 bp). Sequences were aligned using Sequencher 4.1 and those sequences that matched identically were defined as unique lineages. For a subset of the samples for which we obtained mitochondrial sequences, we also amplified a portion of the nuclear genes dihydrofolate reductase–thymidylate synthase (DHFR–TS; 236 bp; Bensch et al., 2004) and diacylglycerol O-acyltransferase (transferase). We amplified the latter with primers trF1 (5′-GCC WAC TAT GTG TTT TCA ATT-3′) and either trR1 (5′-GCT AAM ATA TTY CAC CAA TGA TGA-3′; 294 bp) or trR2 (5′-GGT ATA GAT AAT TTT ARC ATY CTT TC-3′; 231 bp) using conditions identical to those used in amplifying mitochondrial DNA but with an annealing temperature of 50 °C.

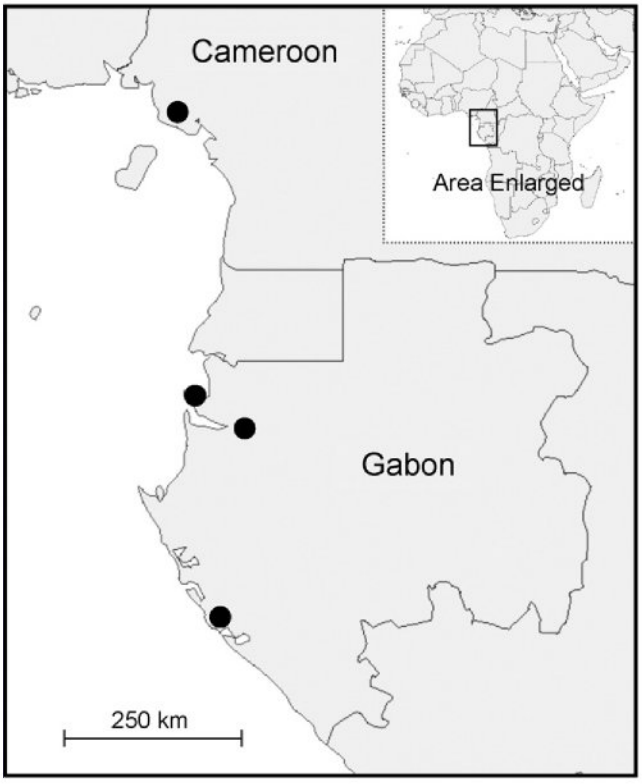

Fig. 1. Map indicating sampling locations within West Africa.

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005

2.2. Phylogenetics

We used a neighbor-joining tree to confirm the identification of parasite mitochondrial lineages to genus (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) based on their association with GenBank sequences from morphologically identified specimens. Subsequently, for lineages of *Plasmodium* spp. and *Haemoproteus* spp. for which we had recovered at least 351 bp of sequence, we used PAUP* (Swofford, D.L., 1998. PAUP*, phylogenetic analysis using parsimony (*and other methods). Version 4.0. Sinauer, Sunderland, MA) to estimate phylogenetic relationships within genera using maximum likelihood (ML) and a general time reversible (GTR) model of nucleotide substitution, which was chosen using the program MODELTEST (Posada and Crandall, 1998). Because these genera appear to be sister taxa (Perkins and Schall, 2002; Martinsen et al., 2008), we rooted the *Plasmodium* spp. tree with *Haemoproteus* spp. and vice versa. For both trees, we estimated support for branches based on 300 bootstrap replicates.

In order to explore the evolutionary relationships among certain lineages of *Plasmodium* more closely, we also generated phylogenetic trees based on DHFR and transferase haplotypes. As a possible consequence of lower copy number, we were not able to amplify nuclear gene fragments from many of the parasites for which we had recovered a mitochondrial signature. In addition, we excluded nuclear sequences for samples in which we detected any evidence of multiple infection (e.g., multiple peaks in the chromatograms obtained for either nuclear sequences or the mitochondrial sequence) in order to help ensure that sequences derived from the same parasite. We estimated relationships using ML and either a GTR (DHFR) or HKY (Hasegawa et al., 1984; transferase) model of nucleotide substitution. Bootstrap support was estimated based on 500 replicates.

2.3. Host specificity analysis

We evaluated the host associations of parasite lineages at multiple levels of parasite relatedness. In all analyses, we defined host specificity both with respect to host species and host family. For avian family definitions, we followed classifications outlined in the Handbook of the Birds of the World (del Hoyo et al., 2003). Initially, we asked whether the host ranges of individual lineages of either *Haemoproteus* or *Plasmodium* were consistent with the random assignment of host species or host families to parasite lineages. To determine the significance of the observed constraint for each lineage, we randomly re-assigned hosts to parasite lineages 1000 times and then compared the observed number of host species or families in which a particular lineage was found with the numbers obtained from randomization. During randomization, we preserved the observed number of times each parasite lineage was detected. We tested for a difference in the number of significantly constrained lineages between the genera *Haemoproteus* and *Plasmodium* using a chi-squared test. For each parasite genus, we also compared the average observed number of host species or host families per parasite lineage with the average obtained from randomizations. We tested whether the average number of hosts per lineage of *Haemoproteus* and *Plasmodium* was different using a Wilcoxon–Mann–Whitney test.

In order to determine whether spillover of parasite lineages into atypical hosts in ecological time may be obscuring longer-term evolutionary signals of host specificity, we compared the host distribution of individual parasite lineages that were detected in two or more host individuals (branch tips) with the host distribution of pairs of sister lineages (i.e., lineages joined by first-step nodes with bootstrap support > 70%). At each level of relatedness (branch tips and first step nodes), we calculated the proportion of parasite lineages occurring in a single host species or in a single host family, and we tested for a significant difference in this metric between parasite genera using a chi-squared test or Fisher's exact test when appropriate.

In order to probe host specificity at deeper levels within the parasite phylogenetic tree, we subsequently performed a logistic regression of host species (same or different) or host family (same or different) versus LogDet parasite distance (Ricklefs and Fallon, 2002; Beadell et al., 2004). We used only lineages with at least 351 bp of sequence data and missing data was ignored when calculating pairwise distances between sequences of different length (PAUP*). Each parasite lineage was included in the dataset once for every time it was detected; therefore the representation of a lineage in a particular host species was proportional to the frequency with which it occurred in that particular host. Thus, a lineage that occurred, for example, four times in one host species and just once in a second host species (e.g., WAH21), would generate a greater signal of host specificity than a lineage occurring just once in each host species (e.g., WAH10). Because multiple pairwise distance calculations violate assumptions of independence, we determined the significance of regression coefficients following a permutation protocol described previously (Beadell et al., 2004). We randomly re-assigned host individuals to the parasite phylogeny 1000 times (again preserving the total number of individuals in which a lineage was observed and the observed number of infected individuals of each host species), performing logistic regression upon each iteration. We then tested for significance by comparing the observed regression coefficients with the distribution obtained from randomization. We tested for a significant regression at the level of host species and at the level of host family in both *Haemoproteus* spp. and *Plasmodium* spp., as well as in several smaller groups of parasite lineages within each genus.

3. Results

3.1. Prevalence

We screened a total of 527 individual hosts representing 93 species and 29 avian families. We detected *Haemoproteus* spp. in 119 individuals (23%), *Plasmodium* spp. in 238 individuals (45%) and *Leucocytozoon* spp. in 35 individuals (7%). In several cases, our methods (sequencing or restriction fragment analysis) failed to identify the parasite genus and these infections were classified as "genus unknown". Prevalence of parasites in well-represented (>20 individuals) avian families is summarized in Table 1. Data for each host species examined is summarized in Supplementary Table S1. Because we recovered *Leucocytozoon* only infrequently, we restricted further analyses to the two most common parasite genera, *Haemoproteus* and *Plasmodium*.

3.2. Phylogenetics

We obtained sequence information from a total of 84 *Haemoproteus* infections (38 unique lineages) and 171 *Plasmodium* infections (47 unique lineages; GenBank accession numbers are listed in Supplementary Table S2). We recovered between one and eight parasite lineages from individual host species, corresponding to an average of 2.1 parasite lineages per species of infected host. For one lineage of *Haemoproteus* (one infection) and five lineages of *Plasmodium* (six total infections), we were unable to recover sequence of at least 351 bp; therefore these lineages were excluded from subsequent analyses requiring a phylogenetic framework. Phylogenetic relationships between mitochondrial lineages of *Plasmodium* spp. and *Haemoproteus* spp. are presented in Figs. 2 and 3, respectively.

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005

Table 1
Prevalence of haematozoan parasites across selected avian host families

Host Family	Species (*n*)	Samples (*n*)	*Haemoproteus*		*Plasmodium*		*Leucocytozoon*		Genus unknown	
			Infected (*n*)	(%)	Infected (*n*)	(%)	Infected (*n*)	(%)	Infected (*n*)	(%)
Alcedinidae	7	32	17	53	1	3	0	0	0	0
Estrildidae	5	24	4	17	8	33	0	0	0	0
Monarchidae	3	21	9	43	2	10	0	0	0	0
Muscicapidae	5	20	4	20	11	55	1	5	1	5
Nectariniidae	12	73	19	26	29	40	2	3	0	0
Ploceidae	6	35	19	54	17	49	4	11	0	0
Pycnonotidae	12	122	9	7	67	55	18	15	2	2
Turdidae	7	67	7	10	61	91	3	4	2	3

A ML tree of *Plasmodium* lineages revealed only one large well-supported clade (PA). Several hosts infected by parasites within clade PA were also infected by distantly related parasites falling outside of this clade, suggesting that these hosts have been subject to multiple independent colonization events. If diversification of parasites can be attributed to both vicariance and host-switching, then signals of host specificity for clades of parasites arising from different host-switching events are likely to coalesce to different points in time. Therefore, when analyzing host specificity (see below) we investigated signals of host specificity arising from several partitions: the entire genus *Plasmodium*, *Plasmodium* excluding clade PA and clade PA alone. Similarly, within *Haemoproteus*, we identified one well-supported sub genus-level clade (HA) which we also examined separately from the remaining parasites. Interestingly, clade HA encompassed most of the *Haemoproteus* lineages derived from non-passerines (Alcedinidae, Indicatoridae, Capitonidae and Strigidae) in our sample.

Evolutionary relationships of parasite lineages deduced from two nuclear genes were similar to those estimated from mitochondrial sequences (data not shown). Our analyses were limited because we recovered nuclear data from only a small subset of mitochondrial lineages and bootstrap support for phylogenetic relationships was low due to the small size of the nuclear fragments targeted. Nonetheless, a plot of linkage between mitochondrial and nuclear markers illustrates the strong correspondence amongst these markers and provides support for defining parasite taxonomic units using mitochondrial lineages alone (Fig. 4). As evidenced in Fig. 4, few squares are shaded outside of the boxes outlined along the diagonal, indicating that the mitochondrial lineages examined are likely to represent reproductively-isolated units. The few exceptions are largely attributable to the sharing of nuclear haplotypes between closely related mitochondrial lineages.

3.3. Host specificity

3.3.1. Host specificity at tips of phylogeny

Assessing lineages individually, we found that of the *Plasmodium* lineages that were testable (i.e., recovered at least twice), three of 23 occurred in significantly fewer host species, and two of 23 occurred in significantly fewer host families than expected by chance (assessed at Bonferroni corrected $P < 0.0022$). For *Haemoproteus* spp., seven of 20 lineages occurred in significantly fewer host species, and three of 20 lineages occurred in significantly fewer families than expected by chance (Bonferroni corrected $P < 0.0025$). The number of lineages exhibiting significant constraint at the host species level ($\chi^2 = 2.89$, df = 1, $P = 0.089$) or at the host family level ($\chi^2 = 0.41$, df = 1, $P = 0.52$) was not significantly higher for *Haemoproteus* spp. compared with *Plasmodium* spp. Individual lineages exhibiting significant constraint at either the host species or family level are identified in Figs. 2 and 3.

Individual lineages of *Haemoproteus* ($n = 37$) were found in an average of 1.3 ± 0.7 (SD) host species and 1.2 ± 0.6 host families (Table 2). Both values were significantly lower ($P < 0.001$) than expected if hosts were randomly distributed across parasite lineages. Similarly, on average, lineages of *Plasmodium* ($n = 42$) were found in significantly fewer species (average = 2.5 ± 4.4, $P < 0.001$) and fewer families (average = 1.6 ± 1.8, $P < 0.001$) than expected by chance. When all lineages were included, Wilcoxon–Mann–Whitney tests revealed no significant differences between either the average number of host species ($Z = -1.67$, $P = 0.096$) or average number of host families ($Z = -0.66$, $P = 0.5083$) infected by lineages of *Haemoproteus* and lineages of *Plasmodium*. However, when considering only those lineages that were detected in two or more individuals (i.e., lineages that were not constrained to just a single host species or family by default), the difference between the average number of host species in which lineages of *Haemoproteus* (1.6 ± 0.8, $n = 20$) and *Plasmodium* (3.7 ± 5.6, $n = 23$) were found was significant ($Z = -2.0$, $P = 0.045$). Again, no significant difference in specificity was observed between *Haemoproteus* and *Plasmodium* at the level of host family ($Z = -0.76$, $P = 0.45$).

3.3.2. Host specificity at greater depth within phylogeny

The proportion of *Haemoproteus* parasite lineages that were detected at least twice, yet were found in just a single host species (60%, $n = 20$), was higher than in *Plasmodium* (30%, $n = 23$) although the difference was only marginally significant ($P = 0.052$; Table 3). No significant difference was apparent when comparing the host species constraint of lineages joined by first-step nodes. At the family level of host specificity, no significant differences were observed between *Haemoproteus* spp. and *Plasmodium* spp., either when comparing identical lineages at branch tips (average divergence = 0%) or lineages joined by well-supported first-step nodes (average divergence = 1.9% for *Haemoproteus* and 1.4% for *Plasmodium*). In both *Haemoproteus* and *Plasmodium* spp., the proportion of lineages constrained to a single host family tended to be higher than those constrained to a single host species when assessed at either branch tips or first-step nodes (Table 3).

Logistic regressions of host species or family versus parasite genetic distance allowed us to extend our analysis of host specificity to even greater depths within the parasite phylogeny. We performed separate logistic regressions on *Haemoproteus* spp. and *Plasmodium* spp. and on two subsets of lineages within each genus. These subsets were composed of either (i) one well-supported clade of parasites nested within each genus (Clade HA or PA) or (ii) all of the remaining lineages in that genus (Group HB or PB). Coefficients generated for all regressions were significant ($P < 0.01$) compared with 1000 randomizations of the data and the resulting trends are presented in Fig. 5. As an index of the phylogenetic depth at which a signal of host specificity was lost (i.e., the genetic distance at which parasites were no longer likely to be found in the same host taxa), we solved logistic regression equations ($\ln(P/(1-P)) = a + b \times x$) obtained for each subset for the distance (x) at which there was a 0.5 probability that two parasite lineages would be found in the same host (Table 4). *Haemoproteus*

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005

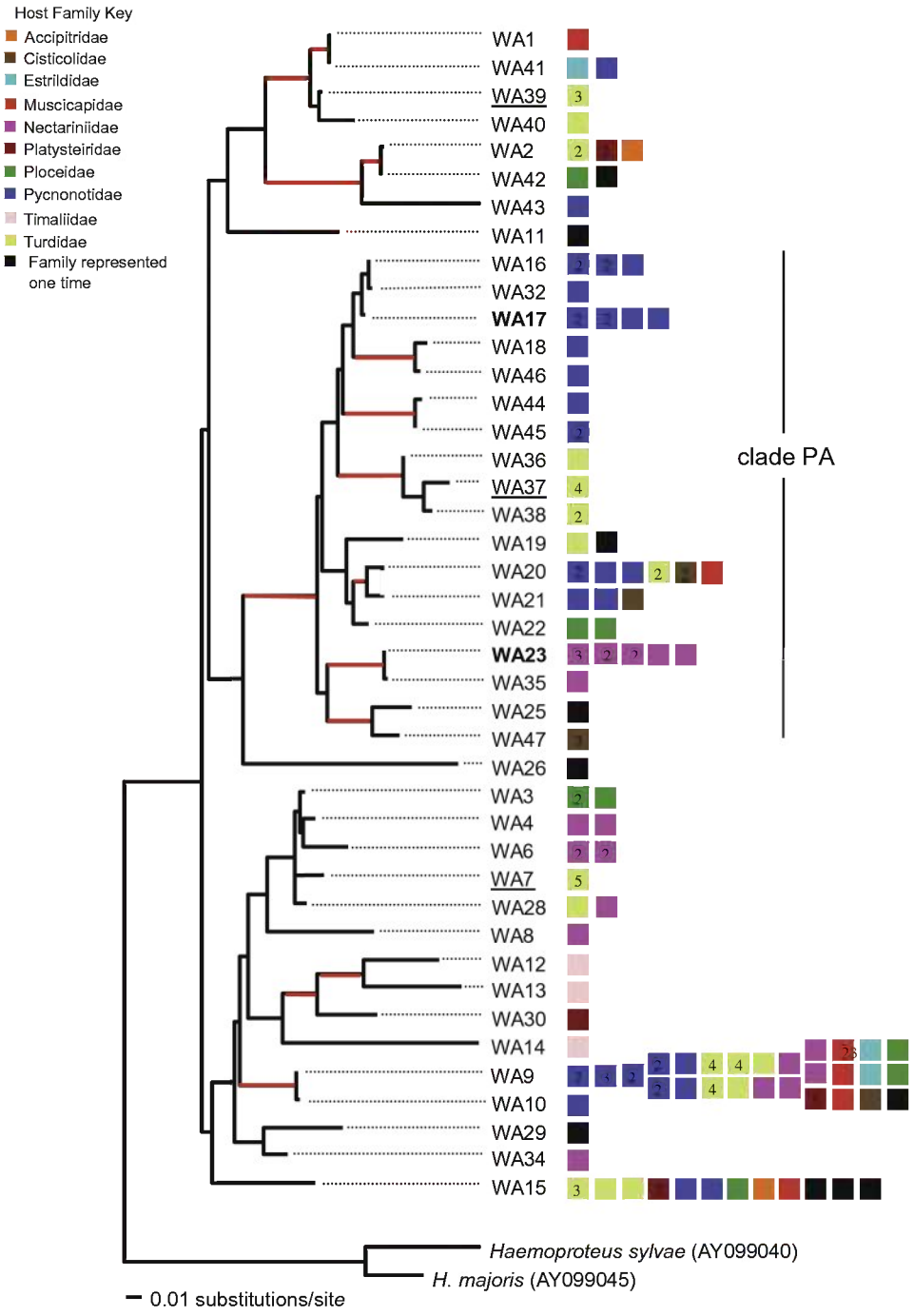

Fig. 2. Evolutionary relationships among *Plasmodium* mitochondrial lineages estimated using maximum likelihood. Branches with bootstrap support > 70% are highlighted in red. Squares to the right of lineage names indicate unique host species in which a particular lineage was recovered and are color coded to indicate the family to which the host species belongs. Numbers within squares indicate the number of host individuals infected. Parasite lineages exhibiting significantly higher specificity at the host species or host family level than expected by chance are indicated by bold or underlined type, respectively.

spp. exhibited a signal of host specificity at the host family level when the genus was examined in its entirety ($x = 0.015$), as well as when partitioned into subsets ($x = 0.013$ for clade HA and $x = 0.017$ for the entire genus minus clade HA). The signal at the host species level was lost at smaller parasite divergences ($x = 0.001$ for the entire genus and $x = 0.005$ for the entire genus minus clade HA). In contrast, only one *Plasmodium* subset (clade PA) exhibited a measurable signal of host specificity ($x = 0.026$) and this was at the level of host family. In all cases, the signal of host specificity was generally stronger at the level of host family than at the level of host species.

4. Discussion

Our results demonstrate subtle differences in the host strategies of *Haemoproteus* spp. and *Plasmodium* spp. and corroborate patterns emerging from previous regional surveys that have revealed broad variability of host–parasitism strategies employed by both genera of avian haematozoa (Beadell et al., 2004; Ricklefs et al., 2004; Fallon et al., 2005; Krizanauskiene et al., 2006). Application of methods similar to those described here to a haematozoan parasite fauna from Australia and Papua New Guinea indicated that lineages of *Haemoproteus* generally appeared

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005

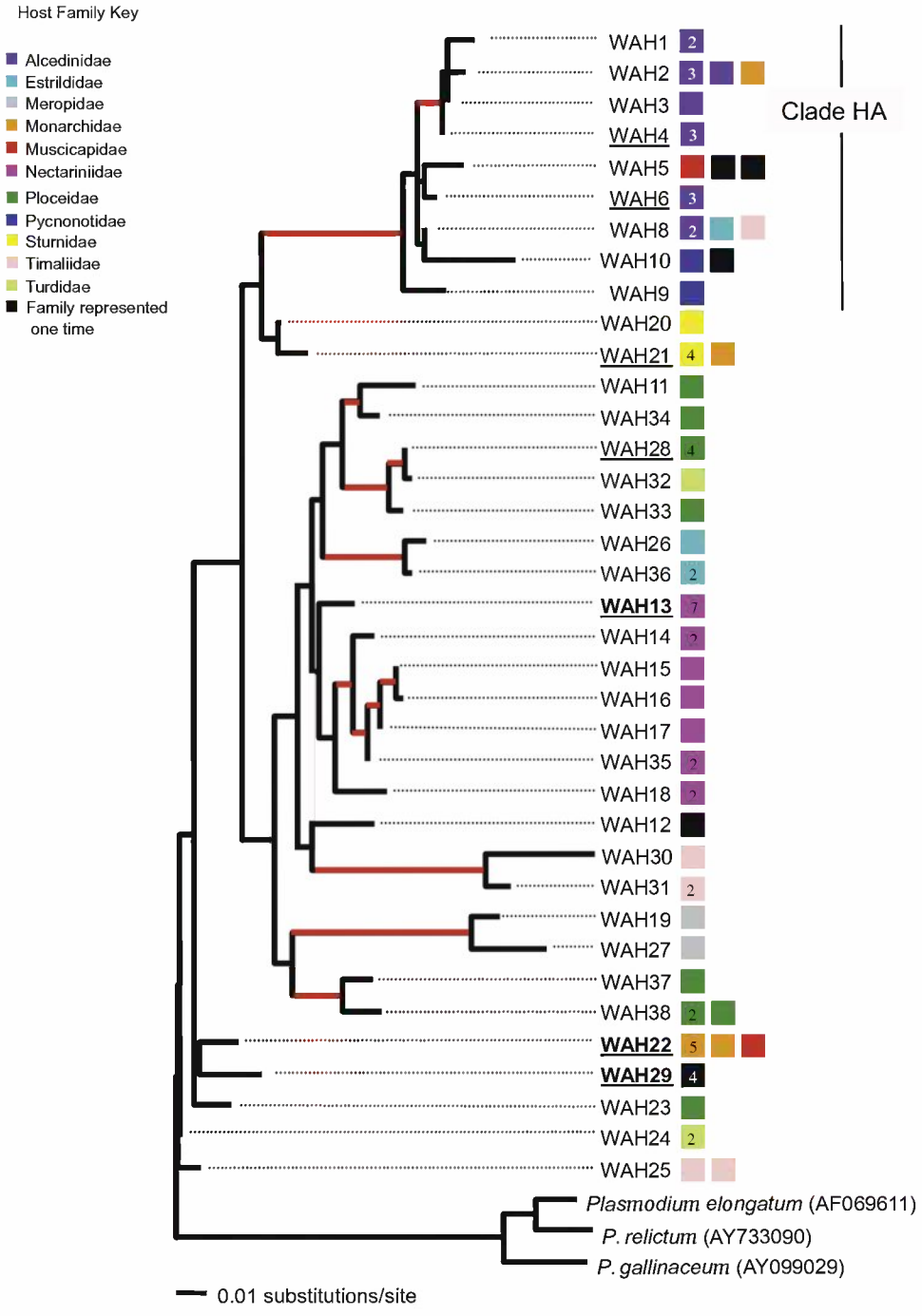

Fig. 3. Evolutionary relationships among *Haemoproteus* mitochondrial lineages estimated using maximum likelihood. Branches with bootstrap support > 70% are highlighted in red. Squares to the right of lineage names indicate unique host species in which a particular lineage was recovered and are color coded to indicate the family to which the host species belongs. Numbers within squares indicate the number of host individuals infected. Parasite lineages exhibiting significantly higher specificity at the host species or host family level than expected by chance are indicated by bold or underlined type, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

to be more constrained at the family level than lineages of *Plasmodium* (Beadell et al., 2004). In the present study we demonstrated that, on average, lineages of *Haemoproteus* were more constrained at the level of host species than were lineages of *Plasmodium*. This difference in host specificity, however, was not evident following removal of the three lineages of *Plasmodium* with the broadest host distribution (WA20, WA9 and WA15). Furthermore, the difference in specificity did not extend to the level of host family. In fact, probing host–parasite associations across the parasite phylogeny using logistic regression suggested that at least one large collection of *Plasmodium* lineages (clade PA) exhibited a signal of host specificity at the host family level (0.026) that was equivalent to the signal obtained for a well-defined group of Australo-Papuan *Haemoproteus* lineages (0.029) and stronger than any signal recovered for groups of West African *Haemoproteus* (range = 0.013–0.017). Thus, at least some lineages of *Plasmodium* appear to be constrained to certain host groups to the same extent as lineages of *Haemoproteus*.

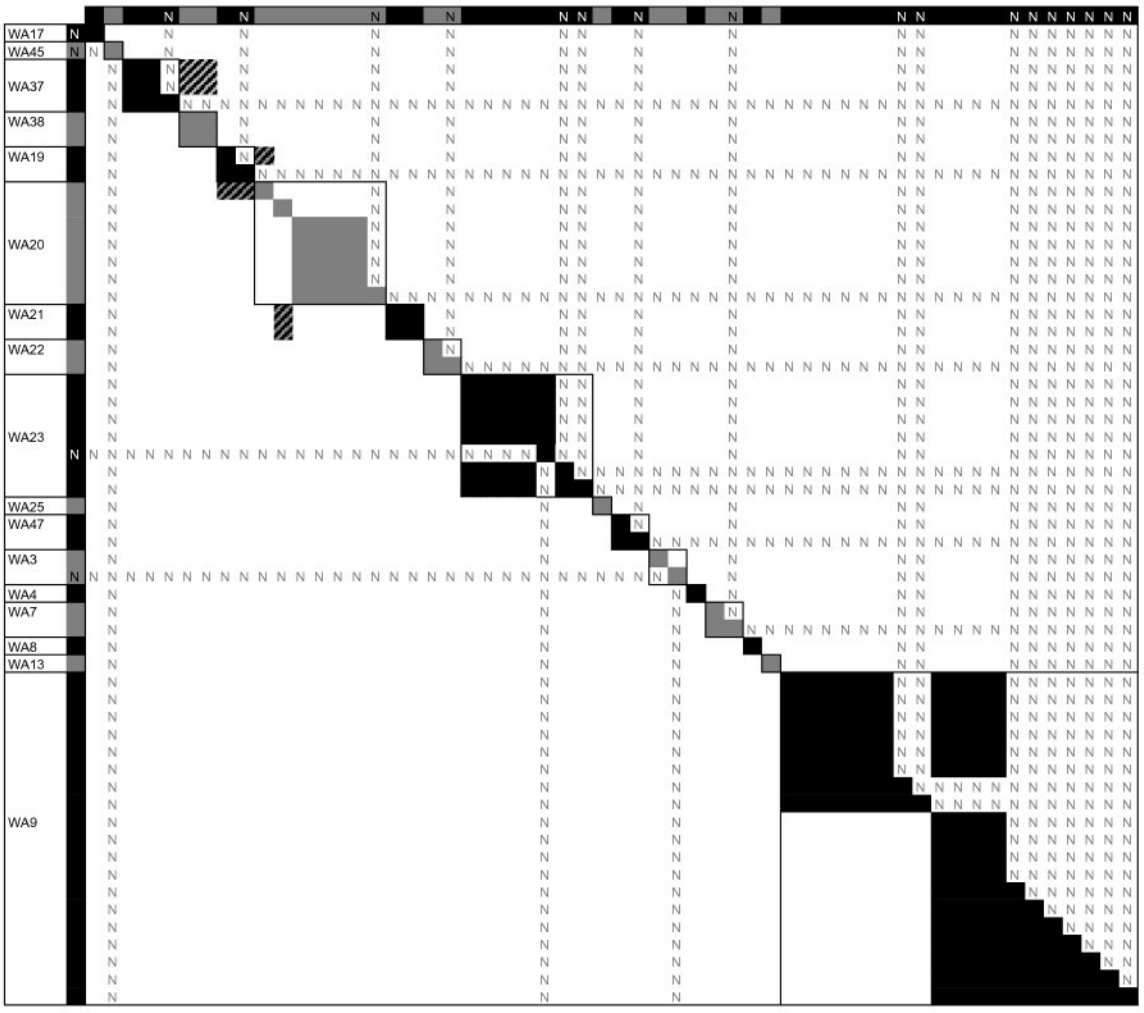

Fig. 4. Linkage plot. Shaded squares indicate sharing of nuclear haplotypes (dihydrofolate reductase–thymidylate synthase (DHFR–TS) below the diagonal; transferase above the diagonal) between individual parasite mitochondrial lineages (identified at left and depicted with corresponding alternate shading in the same order across top). Boxes outlined along the diagonal indicate the squares that would be shaded if each mitochondrial lineage were associated with a unique nuclear signature. Hashed squares indicate sharing of nuclear haplotypes among different mitochondrial lineages. In some cases, identification of shared nuclear haplotypes was not possible (N) due to unsuccessful amplifications of one locus or the other in certain samples.

Table 2
Average host constraint observed across lineages of avian blood parasites

	Haemoproteus	*Plasmodium*
Host species per lineage	1.3 (1.6)	2.5 (3.7)
Host families per lineage	1.2 (1.4)	1.6 (2.1)

Values in parentheses were obtained from averaging across only those lineages that were detected more than once.

Table 3
Host-specificity of haematozoan parasites indicated by the proportion of parasite lineages which derived from the same host species or the same host family at two levels of parasite relatedness (branch tips and first-step nodes)

Parasite genus	Lineages at branch tips			Lineages joined by 1st-step nodes		
	Same host species (%)	Same host family (%)	*n*	Same host species (%)	Same host family (%)	*n*
Haemoproteus	60	70	20	43	86	7
Plasmodium	30	61	23	22	44	9

Because of the extreme variability in observed host–parasitism strategies, particularly in certain lineages of *Plasmodium*, comparing average strategies among haematozoan genera may not be valuable. We recovered individual lineages of *Plasmodium* from between one and 27 different avian host species. The extreme diversity of hosts observed for a single lineage is in keeping with the 39 species of host infected by lineage GRW4 worldwide (Beadell et al., 2006) and the 27 hosts infected by lineage PA in the Antilles (Fallon et al., 2005). We recovered lineages of *Haemoproteus* from a maximum of three host species (three families), but other regional surveys have detected certain lineages of *Haemoproteus* in up to seven (Krizanauskiene et al., 2006) and even 26 (Fallon et al., 2005) host species, suggesting that some *Haemoproteus* lineages may exhibit similarly broad host distributions. In contrast to these generalist lineages, we also identified at least several individual lineages that exhibited significant host constraint at both the host species and host family level. More intensive and thorough sampling of the West African avifauna will undoubtedly expand the host ranges of many of the apparent specialist haematozoan lineages, but the signals of host specificity extending deeper within the *Haemoproteus* phylogeny suggest that many of these lineages are likely to be true specialists. Thus, both *Haemoproteus* and *Plasmodium* appear to harbor lineages with strongly divergent host–parasitism strategies.

Why do related parasites exhibit such striking difference in host specificity? As outlined previously, specialists presumably benefit from relatively high fitness in the limited number of hosts that they utilize and may be able to evolve more quickly in response to changes in host defense or physiology. Generalists, on the other

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005

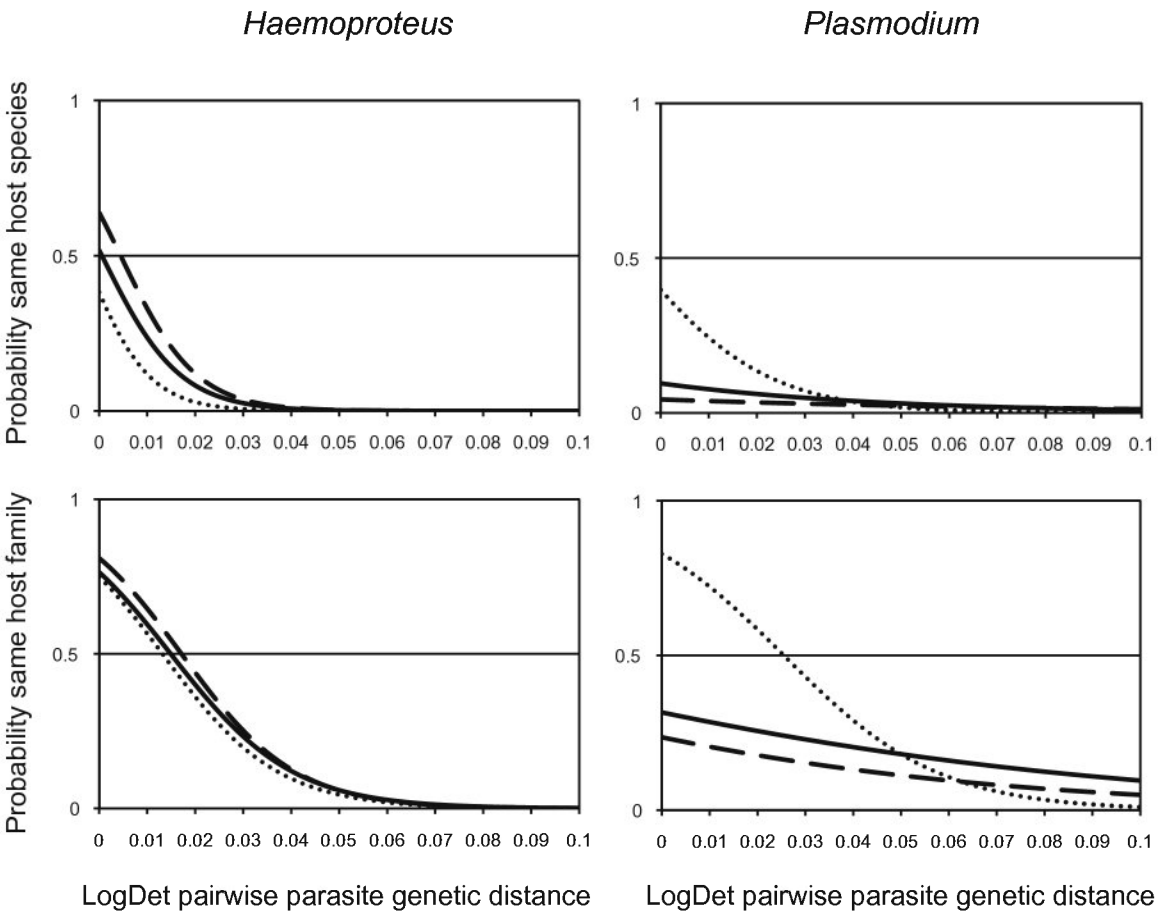

Fig. 5. Logistic regression curves modeling the probability that two parasite lineages exhibit the same host range at different levels of genetic divergence. For both *Haemoproteus* and *Plasmodium*, separate trend lines were calculated using data from the entire genus (black line), a well-supported group of parasites within each genus (HA or PA, dotted line; see Figs. 2 and 3) and the entire genus excluding HA or PA (dashed line). Trend lines that intersect the *y*-axis below 0.5 indicate that, on average, parasite lineages in the group being considered exhibited less than a 50% chance of being found in just a single host species (above) or family (below).

Table 4
Signal of host-specificity derived from logistic regression analysis at various levels of phylogenetic organization within the parasite genera *Haemoproteus* and *Plasmodium*

Phylogenetic partition	Parasite genetic distance at which host-specificity signal lost	
	Host species level	Host family level
Haemoproteus		
Genus	0.001	0.015
Clade HA	n/a	0.013
Genus excluding HA	0.005	0.017
Plasmodium		
Genus	n/a	n/a
Clade PA	n/a	0.026
Genus excluding PH	n/a	n/a

Values indicate the genetic distance beyond which two sampled parasites lineages have less than a 50% chance of deriving from the same host species or same host family. Larger values are indicative of a longer history of association between hosts and parasites.

hand, may be less prone to extinction because they maintain larger populations distributed over a greater number of hosts. Thus, the strategy adopted by a parasite represents a fine balance between the selective pressures favoring either specialist or generalist strategies (Woolhouse et al., 2001). Consequently, host–parasitism strategies may shift rapidly so that even closely related parasites may exhibit very different host ranges. Alternatively, it is possible that the generalist strategy does not exist, or that the generalist strategy represents an unstable and ephemeral transition state (Stireman, 2005). Large observed haematozoan host ranges may reflect the spillover of parasites into hosts in which the full transmission cycle may never be completed. Or, they may simply reflect our inability to distinguish between cryptic parasite species. This phenomenon could explain the extreme lack of specialization observed in *Plasmodium* lineage WA9, detected in 27 different species representing nine host families, and lineage WA15 which was detected in 15 species also representing nine different host families. We cannot rule out the possibility that these lineages actually represent a group of species that are so young that mutations have not yet accumulated to allow differentiation of the numerous specialists that may compose the group.

If mitochondrial lineage diversity is generated too slowly to reflect host specialization, one might question whether mitochondrial lineages are a useful taxonomic unit for investigating host specificity. Certainly, we may be limited in our ability to distinguish recently evolved parasite species. In this case, the broad host ranges of parasite lineages such as WA9 or WA15 may place a downward bias on our estimates of host-specificity. But, at the other end of the spectrum, we may ask whether mitochondrial lineages are valid proxies for long-established reproductively-isolated parasite species. If not, how does this affect estimates of host specificity?

Evidence from two nuclear genes suggests that mitochondrial lineages do provide a reasonable taxonomic metric, at least at the level of mitochondrial differentiation examined here. Although

we cannot determine whether lineages that share a given mitochondrial haplotype are currently reproductively-isolated, sequencing of fragments of the DHFR–TS and transferase genes provided no evidence to refute the common ancestry of parasites sharing a single mitochondrial signature. In other words, the apparent generalism attributed to WA9, for example, did not appear to be the consequence of a single mitochondrial lineage having introgressed into multiple evolutionarily distinct parasites. Nuclear sequences from individuals possessing the WA9 mitochondrial signatures were either identical (transferase) or differed by a single nucleotide (DHFR), suggesting that the parasites sharing this mitochondrial lineage also share a similar nuclear genome.

Evaluated more broadly across other *Plasmodium* lineages, the correspondence between mitochondrial haplotype and nuclear sequences provided support for the use of mitochondrial lineages as the taxonomic basis for evaluating host associations. Mitochondrial lineages tended to be associated with distinct nuclear genotypes; however, the correspondence was not perfect. In at least one case (WA20), a single mitochondrial lineage encompassed three distinct nuclear signatures, evident in both transferase and DHFR. If this mitochondrial lineage is really composed of three reproductively-isolated species, then the host range of this seemingly generalist lineage could be inflated. In several other cases, we identified distinct mitochondrial lineages that shared at least one nuclear haplotype (e.g., WA37 and WA38 (transferase), WA19 and WA20 (DHFR and transferase), WA20 and WA21 (DHFR)). This sharing may represent instances of incomplete lineage sorting in which mitochondrial lineages do actually represent reproductively-isolated species. Alternatively, this sharing of nuclear haplotypes could indicate that the associated mitochondrial lineages simply represent intraspecific diversity. In this case, artificially separating these lineages would inflate estimates of host specificity. For lineages WA37 and WA38, the distinction is irrelevant given that both lineages were found in the same host species. Similarly, changes in host distributions arising from the genetic associations of lineages WA19, WA20 and WA21 would not dramatically alter the signal of host generalism arising from these lineages.

The apparent validity of using mitochondrial lineages as a foundation for investigating host–parasite associations is due in part to the resolution provided by our opportunistic sampling. Among the lineages that we sampled, the average genetic distance between pairs of most closely-related *Plasmodium* lineages (first-step nodes) was about 1.4%. For *Haemoproteus* lineages, the average minimum divergence was about 1.9%. As points of reference, the well-defined and closely-related species *Plasmodium falciparum* and *Plasmodium reichenowi* exhibit a divergence of about 2.3% across the mitochondrial genome (Joy et al., 2003), while morphospecies of *Haemoproteus* can exhibit as little as 0.7% divergence (Hellgren et al., 2007a,b). Mean intra-morphospecies divergence can be substantially higher but at least some of this divergence may represent differentiation among cryptic biological species that share a similar morphology (Beadell et al., 2006; Hellgren et al., 2007a,b). Therefore these data, combined with the strong correspondence between mitochondrial and nuclear haplotypes, suggest that the majority of the parasite lineages in our sample represent species-level taxonomic units. Further integration of genetic and morphological studies (Martinsen et al., 2007; Hellgren et al., 2007a,b), combined with experimental studies of parasite transmission (Iezhova et al., 2005), should help to resolve the species limits of avian blood parasites and lend context to the lineage-level host ranges provided by regional surveys.

Our data suggest that host–parasitism strategies within the genera *Haemoproteus* and *Plasmodium* are variable and can show extreme differences even among closely-related lineages. While we demonstrated that at least some parasites within both genera have been constrained at the level of host-family and even host-species over their evolutionary history, we found evidence of apparent broad host generalism, particularly in certain lineages of *Plasmodium*. Importantly, wide variability in host specificity among lineages of avian haematozoa may be linked to wide variation in virulence (Garamszegi, 2006). Although several studies have demonstrated negative consequences of haematozoan infection for survival, clutch size, incubation period, fledging success, motor activity and fat accumulation (Bennett et al., 1992; Gustafsson et al., 1994; Nordling et al., 1998; Merino et al., 2000; Valkiunas, 2005), few have accounted for possible differences in the virulence of different lineages infecting a particular host population (but see Zehtindjiev et al., 2008). Variability in host specificity, and therefore virulence, of closely related parasite lineages should be accounted for when estimating their impact on host fitness, immunity or life history. Regional surveys have uncovered numerous parasite lineages with extremely divergent host–parasitism strategies; these would now make good candidates for experimentally testing the assumed linkage between host-specificity and virulence.

Acknowledgements

We thank the governments of Cameroon and Gabon for allowing us to conduct our surveys and providing the necessary permits. Fieldwork in Cameroon and Gabon was only possible thanks to the support of Roger Fotso, Joe Mbelle, Okah Monya, Nohou Ndam, Francis Njie, Martin A.E. Ombenotori, Landry Tchignoumba, Patrice Christy and the staff of the Ipassa Research Station. We also thank Liliana Davalos, Eben Gering and Jen Reed for help in the lab. MM was supported by a post-doctoral grant from the French Research Ministry. Fieldwork by CG and BS was supported by the Alexander Wetmore Fund of the Division of Birds, National Museum of Natural History, the Smithsonian Institution's Monitoring and Assessment of Biodiversity (SIMAB) program, Shell Gabon, and the Shell Foundation. Laboratory work was supported by NIH grant GM063258 to RCF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.06.005.

References

Angehr, G., Schmidt, B., Njie, F., Christy, P., Gebhard, C., Tchignoumba, L., Ombenotori, M.A.E., 2006. Bird surveys in the Gamba complex of protected areas, Gabon. In: Alonso, A., Lee, M.E., Campbell, P., Pauwels, O.S.G., Dallmeier, F. (Eds.), Gamba, Gabon: biodiversity of an equatorial African rainforest, vol. 12. Bulletin of the Biological Society of Washington, pp. 327–351.

Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., Atkinson, C.T., Fleischer, R.C., 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Mol. Ecol. 13, 3829–3844.

Beadell, J.S., Fleischer, R.C., 2005. A restriction enzyme-based assay to distinguish between avian hemosporidians. J. Parasitol. 91, 683–685.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y.V., Peirce, M.A., Rahmani, A.R., Fonseca, D.M., Fleischer, R.C., 2006. Global phylogeographic limits of Hawaii's avian malaria. Proc. R. Soc. Lond. Ser. B Biol. Sci. 273, 2935–2944.

Beadell, J.S., Atkins, C., Cashion, E., Jonker, M., Fleischer, R.C., 2007. Immunological change in a parasite-impoverished environment: divergent signals from four island taxa. PLoS ONE 2, e896.

Bennett, G.F., Montgomerie, R., Seutin, G., 1992. Scarcity of haematozoa in birds breeding on the Arctic tundra of North America. Condor 94, 289–292.

Bensch, S., Perez-Tris, J., Waldenstrom, J., Hellgren, O., 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? Evolution 58, 1617–1621.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of

Plasmodium and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. R. Soc. Lond. Ser. B Biol. Sci. 267, 1583–1589.

Bensch, S., Waldenstrom, J., Jonzen, N., Westerdahl, H., Hansson, B., Sejberg, D., Hasselquist, D., 2007. Temporal dynamics and diversity of avian malaria parasites in a single host species. J. Anim. Ecol. 76, 112–122.

Combes, C., Theron, A., 2000. Metazoan parasites and resource heterogeneity: constraints and benefits. Int. J. Parasitol. 30, 299–304.

del Hoyo, J., Elliot, A., Christie, D. (Eds.), 2003. Handbook of the birds of the world. Lynx Edicions, Barcelona.

Durrant, K.L., Beadell, J.S., Ishtiaq, F., Graves, G.R., Olson, S.L., Gering, E., Peirce, M.A., Milensky, C.M., Schmidt, B.K., Gebhard, C., Fleischer, R.C., 2006. Avian hematozoa in South America: a comparison of temperate and tropical zones. Ornithol. Monogr. 60, 98–111.

Durrant, K.L., Reed, J.L., Jones, P.J., Dallimer, M., Cheke, R.A., McWilliam, A.N., Fleischer, R.C., 2007. Variation in haematozoan parasitism at local and landscape levels in the red-billed quelea *Quelea quelea*. J. Avian Biol. 38, 662–671.

Fallon, S.M., Bermingham, E., Ricklefs, R.E., 2005. Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. Am. Nat. 165, 466–480.

Fallon, S.M., Fleischer, R.C., Graves, G.R., 2006. Malarial parasites as geographical markers in migratory birds? Biol. Lett. 2, 213–216.

Garamszegi, L.Z., 2006. The evolution of virulence and host specificity in malaria parasites of primates. Ecol. Lett. 9, 933–940.

Gaunt, A.S., Oring, L.W., Able, K.P., Anderson, D.W., Baptista, L.F., Barlow, J.C., Wingfield, J.C., 1997. Guidelines to the use of wild birds in research. The Ornithological Council, Washington, DC.

Gustafsson, L., Nordling, D., Andersson, M.S., Sheldon, B.C., Qvarnstrom, A., 1994. Infectious diseases, reproductive effort and the cost of reproduction in birds. Philos. Trans. R. Soc. Lond. B 346, 323–331.

Hasegawa, M., Yano, T., Kishino, H., 1984. A new molecular clock of mitochondrial DNA and the evolution of Hominoids. Proc. Japan Acad. Ser. B 60, 95–98.

Hellgren, O., Kristanauskiene, A., Valkiunas, G., Bensch, S., 2007a. Diversity and phylogeny of mitochondrial cytochrome b lineages from six morphospecies of avian *Haemoproteus* (Haemosporidia: Haemoproteidae). J. Parasitol. 93, 889–896.

Hellgren, O., Waldenstrom, J., Perez-Tris, J., Szoll, E., Si, O., Hasselquist, D., Krizanauskiene, A., Ottosson, U., Bensch, S., 2007b. Detecting shifts of transmission areas in avian blood parasites – a phylogenetic approach. Mol. Ecol. 16, 1281–1290.

Iezhova, T.A., Valkiunas, G., Bairlein, F., 2005. Vertebrate host specificity of two avian malaria parasites of the subgenus Novyella: *Plasmodium nucleophilum* and *Plasmodium vaughani*. J. Parasitol. 91, 472–474.

Ishtiaq, F., Beadell, J.S., Baker, A.J., Rahmani, A.R., Jhala, Y.V., Fleischer, R.C., 2006. Prevalence and evolutionary relationships of haematozoan parasites in native versus introduced populations of common myna *Acridotheres tristis*. Proc. R. Soc. Lond. B Biol. Ser. 273, 587–594.

Ishtiaq, F., Gering, E., Rappole, J.H., Rahmani, A.R., Jhala, Y.V., Dove, C.J., Milensky, C., Olson, S.L., Peirce, M.A., Fleischer, R.C., 2007. Prevalence and diversity of avian hematozoan parasites in Asia: a regional survey. J. Wildl. Dis. 43, 382–398.

Joy, D.A., Feng, X., Mu, J., Furuya, T., Chotivanich, K., Krettli, A.U., Ho, M., Wang, A., Suh, E., Beerli, P., Su, X.-Z., 2003. Early origin and recent expansion of *Plasmodium falciparum*. Science 300, 318–321.

Kimura, M., Dhondt, A.A., Lovette, I.J., 2006. Phylogeographic structuring of *Plasmodium* lineages across the North American range of the house finch (*Carpodacus mexicanus*). J. Parasitol. 92, 1043–1049.

Krizanauskiene, A., Hellgren, O., Kosarev, V., Sokolov, L., Bensch, S., Valkiunas, G., 2006. Variation in host specificity between species of avian hemosporidian parasites: evidence from parasite morphology and cytochrome *b* gene sequences. J. Parasitol. 92, 1319–1324.

Martinsen, E.S., Waite, J.L., Schall, J.J., 2007. Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. Parasitology 134, 483–490.

Martinsen, E.S., Perkins, S.L., Schall, J.J., 2008. A three genome phylogeny of malarial parasites (*Plasmodium* and closely related genera): evolution of life history traits and host switches. Mol. Phylogenet. Evol. 47, 261–273.

Merino, S., Moreno, J., Sanz, J.J., Arriero, E., 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc. R. Soc. Lond. B Biol. Ser. 267, 2507–2510.

Nordling, D., Andersson, M., Zohari, S., Gustafsson, L., 1998. Reproductive effort reduces specific immune response and parasite resistance. Proc. R. Soc. Lond. B Biol. Ser. 265, 1291–1298.

Perkins, S.L., Schall, J.J., 2002. A molecular phylogeny of malarial parasites recovered from cytochrome *b* gene sequences. J. Parasitol. 88, 972–978.

Posada, D., Crandall, K., 1998. MODELTEST: testing the model of DNA substitution. Bioinform. 14, 817–818.

Ricklefs, R.E., Fallon, S.M., 2002. Diversification and host switching in avian malaria parasites. Proc. R. Soc. Lond. B Biol. Ser. 269, 885–892.

Ricklefs, R.E., Fallon, S.M., Bermingham, E., 2004. Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. Syst. Biol. 53, 111–119.

Steadman, D.W., Greiner, E.C., Wood, C.S., 1990. Absence of blood parasites in indigenous and introduced birds from the Cook Islands, South Pacific. Conserv. Biol. 4, 398–404.

Stireman, J.O., 2005. The evolution of generalization? Parasitoid flies and the perils of inferring host range evolution from phylogenies. J. Evol. Biol. 8, 325–336.

Taylor, L.H., Latham, S.M., Woolhouse, M.E.J., 2001. Risk factors for human disease emergence. Philos. Trans. R. Soc. Lond. B 356, 983–989.

Valkiunas, G., 2005. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton, Florida.

Wood, M.J., Cosgrove, C.L., Wilkin, T.A., Knowles, S.C.L., Day, K.P., Sheldon, B.C., 2007. Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. Mol. Ecol. 16, 3263–3273.

Woolhouse, M.E.J., Gowtage-Sequeria, S., 2005. Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. 11, 1842–1847.

Woolhouse, M.E.J., Taylor, L.H., Haydon, D.T., 2001. Population biology of multihost pathogens. Science 292, 1109–1112.

Zehtindjiev, P., Ilieva, M., Westerdahl, H., Hansson, B., Valkiunas, G., Bensch, S., 2008. Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp. Parasitol. 119, 99–110.

Please cite this article in press as: Beadell, J.S. et al., Host associations and evolutionary relationships of avian blood parasites from ..., Int. J. Parasitol. (2008), doi:10.1016/j.ijpara.2008.06.005