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A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches

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Abstract

Phylogenetic analysis of genomic data allows insights into the evolutionary history of pathogens, especially the events leading to host switching and diversification, as well as alterations of the life cycle (life-history traits). Hundreds, perhaps thousands, of malaria parasite species exploit squamate reptiles, birds, and mammals as vertebrate hosts as well as many genera of dipteran vectors, but the evolutionary and ecological events that led to this diversification and success remain unresolved. For a century, systematic parasitologists classified malaria parasites into genera based on morphology, life cycle, and vertebrate and insect host taxa. Molecular systematic studies based on single genes challenged the phylogenetic significance of these characters, but several significant nodes were not well supported. We recovered the first well resolved large phylogeny of *Plasmodium* and related haemosporidian parasites using sequence data for four genes from the parasites' three genomes by combining all data, correcting for variable rates of substitution by gene and site, and using both Bayesian and maximum parsimony analyses. Major clades are associated with vector shifts into different dipteran families, with other characters used in traditional parasitological studies, such as morphology and life-history traits, having variable phylogenetic significance. The common parasites of birds now placed into the genus *Haemoproteus* are found in two divergent clades, and the genus *Plasmodium* is paraphyletic with respect to *Hepatocystis*, a group of species with very different life history and morphology. The *Plasmodium* of mammal hosts form a well supported clade (including *Plasmodium falciparum*, the most important human malaria parasite), and this clade is associated with specialization to *Anopheles* mosquito vectors. The *Plasmodium* of birds and squamate reptiles all fall within a single clade, with evidence for repeated switching between birds and squamate hosts.

Keywords: Phylogeny/malaria; Life cycles; Vector shifts; *Plasmodium*; *Haemoproteus*; *Parahaemoproteus*; *Hepatocystis*; *Leucocytozoon*

1. Introduction

Malaria parasites rightfully bear a morbid reputation. Human malaria kills an appalling two million people each year and sickens a half billion others (Teklehaimanot and Singer, 2005). Four species of *Plasmodium* are the causative agents of this massive public health toll (two other species, with apparently limited distributions, have also recently been recognized as agents of human malaria [Singh et al., 2004; Win et al., 2004]), but these few species present only a glimpse of the systematic and ecological diversity of *Plasmodium* and related genera of parasites. Systematic parasitologists have erected approximately 15 genera within the order Haemosporidia (Phylum Apicomplexa) to contain 500+ described species that infect squamate reptiles, turtles, birds, and mammals, and use at least seven families of dipteran vectors (Levine, 1988). These parasites are distributed in every terrestrial habitat on all the warm continents.

In avian hosts alone, 206 species of haemosporidians have been described from hundreds of bird species and from 16 genera of insect vectors (Valkiunas, 2005), and

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recent gene sequence data suggest that there are thousands of undescribed cryptic species that share convergent morphology with known taxa (Bensch et al., 2004). The diversity of malaria parasites has proven to be an important system for pursuing evolutionary and ecological issues such as speciation (Perez-Tris et al., 2007), coevolution (Charleston and Perkins, 2002; Mu et al., 2005), life-history tradeoffs (Eisen and Schall, 2000; Jovani, 2002; Taylor and Read, 1997), the evolution of virulence (Bell et al., 2006; Schall, 2002), sexual selection (Spencer et al., 2005), and competition and community structure (Fallon et al., 2004; Paul et al., 2002; Schall and Bromwich, 1994). Malaria parasites also can have profound conservation significance—the introduction of *Plasmodium relictum* to the Hawaiian islands resulted in a catastrophic decline in the endemic avifauna (Atkinson et al., 2000; van Riper et al., 1986).

For a century, recovering the evolutionary history of *Plasmodium* and its relatives has remained a perplexing (and provocative) problem for systematic parasitologists (Garnham, 1966; Valkiunas, 2005). Even how to define the common colloquial term "malaria parasite" remains an open controversy (Perez-Tris et al., 2005; Valkiunas et al., 2005). Characters used in systematic studies of haemosporidians (both in describing species and defining higher level taxa) classically included morphology seen under the light microscope, life-history traits (variations in the life cycle), and vertebrate and insect host taxon. Table 1 lists the diagnostic characters used to define several of the most common and diverse genera of haemosporidians, and Fig. 1a presents a traditional hypothesis of their evolutionary relationships. However, the phylogenetic significance of the characters used to construct this hypothesis has always been unclear (Garnham, 1966). Morphological characters for taxonomic and systematic studies have primarily been limited to measurements such as the length, width, and shape (e.g. oval, elongated, reniform, etc.) of the life stages present in host erythrocytes (Martinsen et al., 2006). Morphology of stained cells under the microscope examined in a two-dimensional framework can give only a crude indication of the true three-dimensional structure of the parasites (analogous to systematic study of insects based on remains seen on automobile windshields), and has been shown to vary depending on the species of host and age of infection (Jordan, 1975; Valkiunas, 2005). Life-history traits used to define species and genera include the types of host cells that the parasite uses for schizogony (a process of asexual division involving multiple divisions of the nucleus resulting in a mature schizont cell that divides into several to many daughter cells), the num-

Table 1
Genera of malaria parasites and other haemosporidians (Phylum Apicomplexa) included in this study as they are currently defined

Genus	No. of described species	Vertebrate hosts	Vectors (Diptera)	Erythrocytic schizogony	Pigment
Plasmodium	199	M, B, S	Mosquitoes (Culicidae); sandflies (Psychodidae)	Yes	Yes
Haemoproteus	202	B, S	Louse flies (Hippoboscidae); biting midges, (Ceratopogonidae)	No	Yes
Hepatocystis	25	M	Biting midges (Ceratopogonidae)	No	Yes
Leucocytozoon	91	B	Blackflies (Simulidae)	No	No

M, mammals; B, birds; S, squamate reptiles. Species numbers from Levine (1988) and Valkiunas (2005) and updated from the numerous literature references as of July 2007.

These four genera account for most (>90%) of described species of haemosporidians worldwide. Erythrocytic schizogony is the asexual replication of haploid cells (termed meronts or schizonts) within red blood cells. All the parasites replicate asexually at least initially in deep tissues such as the liver. Pigment refers to storage of the waste products of digestion of hemoglobin molecules as crystals within the infected blood cell.

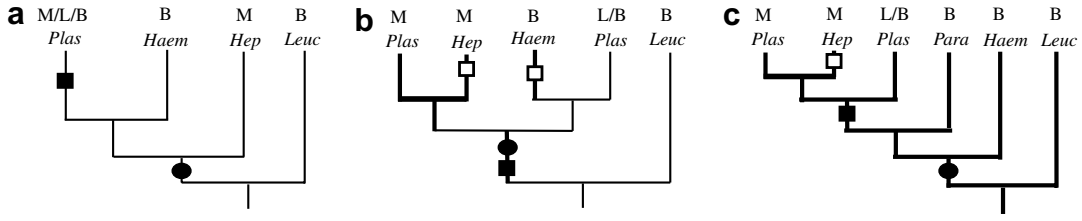

Fig. 1. Comparison of three phylogenetic hypotheses for the relationship for several haemosporidian genera. M, mammalian parasites; L, lizard parasites: B, bird parasites. *Plas*, *Plasmodium*; *Haem*, *Haemoproteus*; *Hep*, *Hepatocystis*; *Leuc*, *Leucocytozoon*; and *Para*, *Parahaemoproteus* (current taxonomic status is a subgenus). Filled squares represent the gain of blood schizogony; open squares represent the loss of this character. Filled circles represent the presence of hemozoin pigment in the cells. (a) The traditional hypothesis is based on morphology and life-history traits of the parasites and was the prevailing view for decades. (b) The one-gene hypothesis is based on analysis of the cytochrome *b* gene from the mitochondrion with lack of strong support for many nodes within the phylogeny (Perkins and Schall, 2002). (c) The four-gene hypothesis is based on the current analysis of four genes from the parasites' three genomes. Strong nodal support is represented by heavy branch lines. Under hypothesis (c) the parasites combined within the genus *Haemoproteus* in hypotheses (a) and (b) are found to be separate clades, the basal *Haemoproteus* and a distinct group *Parahaemoproteus*.

ber of daughter cells produced by each mature schizont, and the presence or absence of hemozoin pigment stored within the parasite cell (the product of the breakdown of hemoglobin, formed by crystallization of the porphyrin). The adaptive significance, if any, in the variation in these life-history traits is unknown, but they could well evolve convergently to respond to ecological changes such as host switches. Last, host switching has long been viewed as a major event in the history of the parasites (Garnham, 1966) because of the different host physiology and cell structure (nucleated erythrocytes in avian and squamate reptiles *vs*. anucleated red blood cells in mammals), and divergent transmission requirements (feeding behavior and ecology of the vectors). However, host switching could occur multiple times independently.

The suspicion that characters used to define higher level taxa of malaria parasites might sometimes be the result of convergence led Manwell (1936) to conclude that only genetic data would provide a clear image of the relationships among haemosporidian taxa. This conclusion was prognostic. Gene sequence data have begun to challenge classical systematic hypotheses for the malaria parasites, both within the genus *Plasmodium*, as well as the relationships among the currently recognized genera (Escalante and Ayala, 1994; Escalante et al., 1995, 1998; Qari et al., 1996; Perkins and Schall, 2002; Martinsen et al., 2007). For example, these studies suggest that the genus *Plasmodium* is paraphyletic relative to *Haemoproteus* and *Hepatocystis*, despite striking differences in life-history traits and morphology of the three recognized genera. *Haemoproteus*, a common parasite of birds almost worldwide, was shown to be polyphyletic within the *Plasmodium* clade suggesting multiple shifts between avian and squamate reptile hosts (Perkins and Schall, 2002). These results thus suggested that the distinctive characteristic defining *Plasmodium*, schizogony in the vertebrate host's erythrocytes, has been secondarily lost several times within the clade. Also, the placement of *Haemoproteus* and *Hepatocystis* within the *Plasmodium* clade argues that vector switches between insects as diverse as mosquitoes (Culicidae), biting midges (Ceratopogonidae), and louse flies (Hippobosidae) also occurred multiple times. These are intriguing results, but are based on analysis of single-gene phylogenies (cytochrome *b*, SSU rRNA, circumsporozoite protein) with many poorly supported nodes. Fig. 1b presents the phylogenetic hypothesis emerging from the single-gene studies (e.g. Perkins and Schall, 2002).

Here we recover a broad multi-gene phylogeny of malaria parasites, including in the analysis sequence data from four genes from the parasites' three genomes, using some published data, but also adding many new sequences for lizard, bird, and mammal parasites for the four loci. We focused on three genera of the order Haemosporidia (*Plasmodium*, *Haemoproteus*, and *Hepatocystis*) that comprise over 90% of the documented haemosporidian species and have the widest host ranges (Table 1; Levine, 1988). Several isolates from a fourth genus (*Leucocytozoon*) were used as outgroup taxa (Perkins and Schall, 2002). Coding regions from four genes were sequenced: two came from the mitochondrion (cytochrome *b*, hereafter *cytb* and cytochrome oxidase I, *coI*), one came from the nucleus (adenylosuccinate lyase, *asl*), and one from the plastid genome (caseinolytic protease, *clpc*). A large sample of ingroup taxa ($N = 57$) from lizard, bird, and mammal hosts worldwide were used, thus reducing the possibility of spurious results due to taxon bias. A previous study compared neighbor-joining trees obtained from analysis of one gene from each of the parasites' genomes for eight *Plasmodium* species; the trees presented very different topologies (Rathore et al., 2001). This is expected if the genes have different rates and patterns of evolution. Recently, another study used sequence data from the three genomes (including *cytb* and *clpc*) and several of the same taxa, but again analyzed the datasets separately (Hagner et al., 2007). In our analyses, we combined all data and used maximum parsimony and Bayesian methods to correct variation that existed gene-by-gene and site-by-site. The new phylogeny has a topology with high support on all important nodes and thus casts light on several standing issues in the evolution of malaria parasites, including the monophyly of *Plasmodium*, convergence in life cycle characters, vector use, and host switching between squamate reptiles and birds for *Plasmodium*.

2. Materials and methods

2.1. Specimens

Data for all primate *Plasmodium* samples were obtained from GenBank. Original samples, always taken from vertebrate blood, were obtained during our field collecting or from other researchers and followed university approved animal care and use protocols. Blood samples to be used for molecular analysis were stored dried and frozen on filter paper. A total of 11 mammalian parasite species, seven species that infect lizards, and 39 lineages that were obtained from avian hosts were included in the analyses. Three samples consistent with morphology of *Leucocytozoon* were used as outgroup taxa. Details on the origin of samples are available in Table 2. With the exception of the primate *Plasmodium* species, all samples were examined as thin blood smears to identify the parasite to species or at least to genus. As the avian *Plasmodium* samples encountered in our study were light in intensity and offered little in the way of diagnostic characters, only a few infections were confidently identified to the level of species (Martinsen et al., 2006, 2007).

2.2. DNA extraction, PCR amplification and sequencing

DNA extraction was performed using the DNeasy kit (Qiagen, USA). Sequences included 1605 nucleotides (nt) of mitochondrial DNA (607 nt of the *cytb* and 998 nt of the *coI* genes), 523 nt of the *clpc* gene, and 206 nt of the

Table 2
Host species, geographic origin, and GenBank Accession numbers for the parasite taxa used in the study

Parasite	Host	Sampling origin	GenBank Accession Nos.
Outgroup taxa			
Leucocytozoon sp.	*Buteo jamaicensis*	Massachusetts, USA	EU254518, EU254563 EU254609, EU254663
Leucocytozoon sp.	*Accipiter brevipes*	Israel	EU25451, EU254564 EU254610, EU254664
Leucocytozoon sp.	*Buteo lineatus*	California, USA	EU254520, EU254565 EU254611, EU254665
Mammal *Plasmodium*			
P. falciparum	Humans	Tropical regions	AF069605, M76611, CAA60961, M
P. vivax	Humans	Brazil	AF069619, AAY26841, AF348344, AAL60072
P. knowlesi	Old World Monkeys	Malaysia	AF069621, AY598141, AF348341, AAL60073
P. yoelii	*Thamnomys rutilans*	Central African Republic	EU254521, EU254566 EU254612, EU254666
P. berghei	*Grammomys surdaster*	Katanga, Congo	EF011166, EF011199 AF348337, EU254670
P. vinckei	*G. surdaster*	Katanga, Congo	EU254522, EU254567 EU254613, EU254667
P. atheruri	*Atherurus africanus*	Katanga, Congo	EU254524, EU254568 EU254615, EU254669
P. chabaudi	*T. rutilans*	Central African Republic	EF011167, EF011200 EU254614, EU254668
Mammal *Hepatocystis*			
Hepatocystis sp.	*Cynopterus brachyoti*	Singapore	EU254526, EU254569 EU254616, EU254671
Hepatocystis sp.	*Nanonycteris veldkampii*	Guinea	EU254527, EU254570 EU254617, EU254672
Hepatocystis sp.	*Nanonycteris veldkampii*	Guinea	EU254528, EU254571 EU254618, EU254673
Lizard/bird *Plasmodium*			
P. mexicanum	*Sceloporus occidentalis*	California, USA	EU254529, EU254572 EU254619, EU254674
P. floridense	*Anolis oculatus*	Dominica	EU254530, EU254573 EU254620, EU254675
P. azurophilum R	*A. oculatus*	Dominica	EU254532, EU254575 EU254622, EU254677
P. azurophilum W	*A. oculatus*	Dominica	EU254533, EU254576 EU254623, EU254678
Plasmodium sp.	*Ameiva ameiva*	Manaus, Brazil	EU254537, EU254580 M, EU254684
P. giganteum	*Agama agama*	Ghana	EU254534, EU254577 EU254624, EU254679
Plasmodium sp.	*Acridotheres tristis*	Singapore	EU254542, EU254585 EU254636, EU254693
P. gallinaceum	*Gallus gallus*	Vietnam	EU254535, EU254578 EU254625, EU254680
P. relictum	*Emberiza hortulana*	Israel	EF011193, EF011226 EU254627, EU254682
Plasmodium sp.	*Corvus corone*	Israel	DQ451404, EU254593 EU254645, EU254701
Plasmodium sp.	*Emberiza hortulana*	Israel	EF011194, EF011227 EU254628, EU254683
Plasmodium sp.	*Spizella passerina*	Vermont, USA	EF011176, EF011209 EU254632, EU254688
P. relictum	*Sialia mexicana*	California, USA	EU254538, EU254581 EU254633, EU254689
P. relictum	Zenaida macroura	Nebraska, USA	EU254536, EU254579 EU254626, EU254681
Plasmodium sp.	*Luscinia svecica*	Israel	EU254540, EU254583 EU254634, EU254691

Table 2 (*continued*)

Parasite	Host	Sampling origin	GenBank Accession Nos.
Plasmodium sp.	*Larosterna inca*	Washington, D.C.	EU254547, EU254590 EU254641, EU254698
Plasmodium sp.	*Melospiza melodia*	Vermont, USA	EF011168, EF011201 EU254629, EU254685
Plasmodium sp.	*Aegolius acadicus*	Vermont, USA	EU254543, EU254586 EU254637, EU254694
Plasmodium sp.	*Accipiter striatus*	Vermont, USA	EU254539, EU254582 M, EU254690
Plasmodium sp.	*Ixobrychus minutus*	Israel	EU254541, EU254584 EU254635, EU254692
Plasmodium sp.	*Agelaius phoeniceus*	Vermont, USA	EF011171, EF011204 EU254630, EU254686
Plasmodium sp.	*Seiurus aurocapilla*	Vermont, USA	EF011173, EF011206 EU254631, EU254687
Plasmodium sp.	*Hylocichla mustelina*	Vermont, USA	EU254544, EU254587 EU254638, EU254695
Plasmodium sp.	*Turdus migratorius*	Vermont, USA	EU254545, EU254588 EU254639, EU254696
Plasmodium sp.	*Anthus trivialis*	Israel	EU254546, EU254589 EU254640, EU254697
Plasmodium sp.	*Egernia stokesii*	Australia	EU254531, EU254574 EU254621, EU254676
Bird *Parahaemoproteus*			
H. syrnii	*Strix selupto*	Israel	DQ451424, EU254591 EU254643, EU254700
H. turtur	*Streptopelia senegalensis*	Israel	DQ451425, EU254592 EU254644, M
H. picae	Picoides pubescens	Vermont, USA	EU254552, EU254597 EU254650, EU254706
Haemoproteus sp.	*Bonasa umbellus*	Vermont, USA	EU254555, EU254600 EU254654, EU254709
Haemoproteus sp.	*Mergus merganser*	Vermont, USA	EU254560, EU254606 EU254660, M
Haemoproteus sp.	*Bucephala clangula*	Vermont, USA	EU254561, EU254607 EU254661, M
H. magnus	*Fringilla coelebs*	Israel	DQ451426, EU254594 EU254647, EU254703
H. fringillae	*Zonotrichia albicollis*	Vermont, USA	EU254558, EU254604 EU254658, EU254711
H. belopolskyi	*Sylvia curruca*	Israel	DQ451408, EU254603 EU254657, EU254710
Haemoproteus sp.	*Vireo olivaceus*	Vermont, USA	EU254551, EU254596 EU254649, EU254705
H. coatneyi	*Dendroica coronata*	Vermont, USA	EU254550, EU254595 EU254648, EU254704
Haemoproteus sp.	*Dendroica caerulescens*	Vermont, USA	EU254562, EU254608 EU254662, M
H. passeris	*Passer moabiticus*	Israel	EU254554, EU254599 EU254653, EU254708
H. sanguinis	*Pycnonotus xanthopygos*	Israel	DQ451410, EU254598 EU254651, M
Haemoproteus sp.	*Chamaea fasciata*	California, USA	EU254557, EU254602 EU254656, M
Haemoproteus sp.	*Dumetella carolinensis*	Vermont, USA	EU254559, EU254605 EU254659, M
Haemoproteus sp.	*Falco sparverius*	Vermont, USA	EU254556, EU254601 EU254655, M
Bird *Haemoproteus*			
H. columbae	*Columba livia*	Massachusetts, USA	EU254548, M EU254642, EU254699
H. columbae	*Columba livia*	Singapore	EU254549, M EU254646, EU254702

(*continued on next page*)

Table 2 (*continued*)

Parasite	Host	Sampling origin	GenBank Accession Nos.
H. columbae	*Columba livia*	Massachusetts, USA	EU254553, M EU254652, EU254707

M, missing gene sequence data for a given gene.
Parasite taxa are listed in the order they appear in the phylogeny in Fig. 2. GenBank Accession numbers are provided in the gene order: cytochrome *b*, cytochrome oxidase subunit I, caseinolytic protease, and adenylosuccinate lyase.

asl gene. Primers, PCR conditions, and sequencing procedures for all genes are presented in Table 3. If sequences revealed ambiguous base calls, the sample was re-amplified and re-sequenced. Continued presence of ambiguous bases suggested a mixed-species infection, and these samples were discarded from the study. Sequences were edited using Sequencher (Genecodes, USA), and aligned by eye using MacClade version 4.05 (Maddison and Maddison, 2002). An indel of three nucleotides was included for the *cytb* gene and two indels of three nucleotides each were added in the alignment of the *clpc* gene sequences.

2.3. Phylogenetic analyses

All four genes were combined for phylogenetic analyses. Three parasites, identified as *Leucocytozoon*, collected from Old World and New World hawk species were used as outgroup taxa to root the trees, because they are closely related to, but not contained within, the *Plasmodium*, *Haemoproteus*, and *Hepatocystis* parasite clades (Perkins and Schall, 2002). Although apicomplexan parasites of the genera *Theileria* or *Toxoplasma* have previously been used to root phylogenies of the malaria parasites (e.g. Escalante et al., 1998; Perkins and Schall, 2002), these outgroup taxa are very distantly related to the ingroup taxa under study (over 30% genetic divergence) and we found in our preliminary results that when these taxa were included, the alignments became problematic because of numerous indels.

For designation of site-specific rate groupings, a maximum parsimony analysis was conducted using PAUP* 4.0b (Swofford, 2002). The four genes were concatenated into a single data matrix and subjected to a fast heuristic bootstrap analysis from which five character rate classes were identified (Kjer et al., 2001). A maximum parsimony tree was generated using the site-specific rate weighting scheme and a heuristic search with 10,000 random additions in PAUP* 4.0b. Nodal support values were generated using 10,000 heuristic bootstrap replications under the site-specific weighting scheme.

For Bayesian phylogenetic inference, two partitioning strategies were employed using MCMC for the four-gene dataset (Ronquist and Huelsenbeck, 2003). The first partition used the five rate classes identified by the maximum parsimony analysis above and the second partitioned the dataset by gene. For each data partition, the model of char-

Table 3
Nested PCR primers and cycle conditions for each gene: cytochrome *b* (*cytb*), cytochrome oxidase subunit I (*coI*), caseinolytic protease (*clpc*), and adenylosuccinate lyase (*asl*)

Gene/PCR	Forward (F) and reverse (R) PCR primers	Cycle conditions: temperature (°C)/time (s) for denaturation, annealing, and extension steps
cytb/outer	F: TAATGCCTAGACGTATTCCTGATTATCCAG R: TGTTTGCTTGGGAGCTGTAATCATAATGTG	94/20, 60/30, 68/50
coI/outer	F: CTATTTATGGTTTTCATTTTTATTTGGTA R: AGGAATACGTCTAGGCATTACATTAAATCC	94/20, 60/30, 68/50
clpc/outer	F: AAACTGAATTAGCAAAAATATTA R: CGWGCWCCATATAAAGGAT	94/30, 45/30, 62/50
asl/outer	F: GSKAARTTTAATGGKGCTGTWGG R: GGATTAAYTTTATGAGGCATTG	94/30, 45/30, 62/50
cytb/nested	F: TCAACAATGACTTTATTTGG R: TGCTGTATCATACCCTAAAG	94/20, 52/20, 68/50
coI/nested	F: ATGATATTTACARTTCAYGGWATTATTATG R: GTATTTTCTCGTAATGTTTTACCAAAGAA	94/20, 52/20, 68/50
clpc/nested	F: GATTTGATATGAGTGAATATATGG R: CCATATAAAGGATTATAWG	94/30, 48/30, 62/30
asl/nested	F: GCTGATMAAAATRTTGATTGG R: GAGGCATTGTACTACTWCC	94/30, 45/30, 62/30

All outer reactions included an initial denaturation period of 4 min at 94 °C and 35 cycles. Nested reactions included an initial denaturation period of 1 min at 94 °C and 40 cycles. All reactions included a final extension period of 7 min at 68 °C.

acter evolution was selected using MrModeltest v2.2 (Nylander, 2004). The GTR + Γ model was selected for the five rate classes and the *cytb* gene and the GTR + I + Γ model for the *coI*, *clpc*, and *asl* genes. We ran five million generations (two independent runs, four chains) with sampling every 100 generations using MrBayes V3.1.2 for each of the two partitioning strategies (Ronquist and Huelsenbeck, 2003). For each data partition, the selected model of sequence evolution was indicated and the appropriate model parameter values were independently estimated. Convergence in phylogeny estimation was assessed for each analysis using the program AWTY and used to indicate the appropriate "burn-in" period (Wilgenbusch et al., 2004).

3. Results

We obtained sequence data for all four genes for most of the samples. For a small number of samples, we were not able to amplify the *coI* ($N = 3$), *clpc* ($N = 2$), or *asl* ($N = 9$) gene. The maximum parsimony and two Bayesian analyses resulted in similar tree topologies, the only difference being that more basal nodes were well supported ($\geqslant 90\%$) and thus resolved by the Bayesian analyses. Bayesian posterior probability values lend strong support for nodes relevant to the questions under study. Only nodes with $\geqslant 90\%$ posterior probability value by both Bayesian analyses are depicted in the tree figures and all relationships reported below were well supported by both Bayesian analyses ($\geqslant 95\%$). Fig. 2 presents the full phylogeny and Fig. 3 is a summary to emphasize major events in the evolution of the parasites. Nodes with bootstrap support values greater than or equal to 90% are indicated in Fig. 2.

The avian *Haemoproteus* fell into two divergent clades. The first was sister to all other ingroup taxa and contained three parasites sampled from doves from North America and Singapore that were morphologically consistent with *Haemoproteus columbae*, a cosmopolitan parasite of doves (Bennett and Peirce, 1990; Valkiunas, 2005). All other species identified as "*Haemoproteus*" from non-columbiform birds formed a sister group to the *Plasmodium* and *Hepatocystis* species in mammals, birds, and lizards. Parasites that were identified as *Plasmodium* species, fell into two well-supported major clades, one containing parasites of mammals, the other parasites of lizards and birds. Within the mammalian parasite clade, which itself was supported by a Bayesian posterior probability of 99%, there are four monophyletic lineages: the human parasite, *P. falciparum*; a "primate" lineage that contains *Plasmodium vivax* and *Plasmodium knowlesi*, a lineage of parasites that infect African rodents, and the three samples of *Hepatocystis* isolated from bats. The *Plasmodium* parasites of bird and lizard hosts do not form two separate clades that are consistent with their host taxa, but instead, fall into eight distinct lineages. Most of these monophyletic groups are consistent with their vertebrate host, but there is one notable exception of a *Plasmodium* species from a bird from Southeast Asia grouping with a clade of lizard malaria parasites. The relationships between these avian and lizard parasite lineages are still not well resolved, preventing an estimate of number of switches between bird and lizard hosts.

4. Discussion

4.1. Three phylogenetic hypotheses and the evolution of life history traits

We compare three phylogenetic hypotheses for the relationships of closely related groups of parasites that are currently placed in the genera *Plasmodium*, *Haemoproteus*, *Hepatocystis*, and *Leucocytozoon*; a traditional hypothesis based on morphology and life history (Fig. 1a), a hypothesis emerging from single-gene molecular studies (Fig. 1b), and a new hypothesis leading from the four gene study with strong nodal support (Fig. 1c). The morphology hypothesis (Fig. 1a) represents the most parsimonious view, that the ability to store hemozoin pigment evolved once, in the ancestor of a large clade containing the sister clades *Plasmodium* and *Haemoproteus* as well as *Hepatocystis*, and asexual replication in erythrocytes evolved once at the origin of the *Plasmodium* clade. *Leucocytozoon* is viewed as sister to the other genera. The single-gene hypothesis (Fig. 1b; Perkins and Schall, 2002) also finds *Leucocytozoon* to be a more distantly related group, with the rest of the parasites grouping into a single clade containing species of *Plasmodium*, *Haemoproteus*, and *Hepatocystis*, implying one gain and several losses of asexual replication in erythrocytes and a single gain of storing hemozoin pigment. In the single-gene tree, *Plasmodium* is viewed as paraphyletic relative to *Haemoproteus* and *Hepatocystis*. The *Plasmodium* parasites of mammals are monophyletic, but those of birds and lizards represent unresolved relationships. The current four-gene hypothesis (Fig. 1c) finds that *Plasmodium* is not paraphyletic relative to the avian *Haemoproteus*, but instead, avian *Haemoproteus* taxa fall into two clades sister to *Plasmodium*. In this hypothesis, there was a single gain of hemozoin storage, and a gain of schizogony in the blood at the origin of *Plasmodium*, with secondary loss of this character in *Hepatocystis*.

4.2. A revised taxonomy of "*Haemoproteus*"

The type species for the genus *Haemoproteus* is *H. columbae*, which the phylogeny reveals is not closely related to most other species of *Haemoproteus*. Some authorities divide avian *Haemoproteus* into two subgenera based on vector hosts, *Haemoproteus* infecting doves, Columbiformes, and vectored by hippobosid flies, and *Parahaemoproteus* infecting a large diversity of species recorded from most other bird families world-wide and vectored by biting midges, Ceratopogonidae (Valkiunas, 2005). The current phylogeny supports the keeping of the parasites infecting columbiform birds and hippoboscid flies within the genus *Haemoproteus* and re-classifying the greater diversity of *Haemoproteus* of other birds and biting midges as *Parahae-*

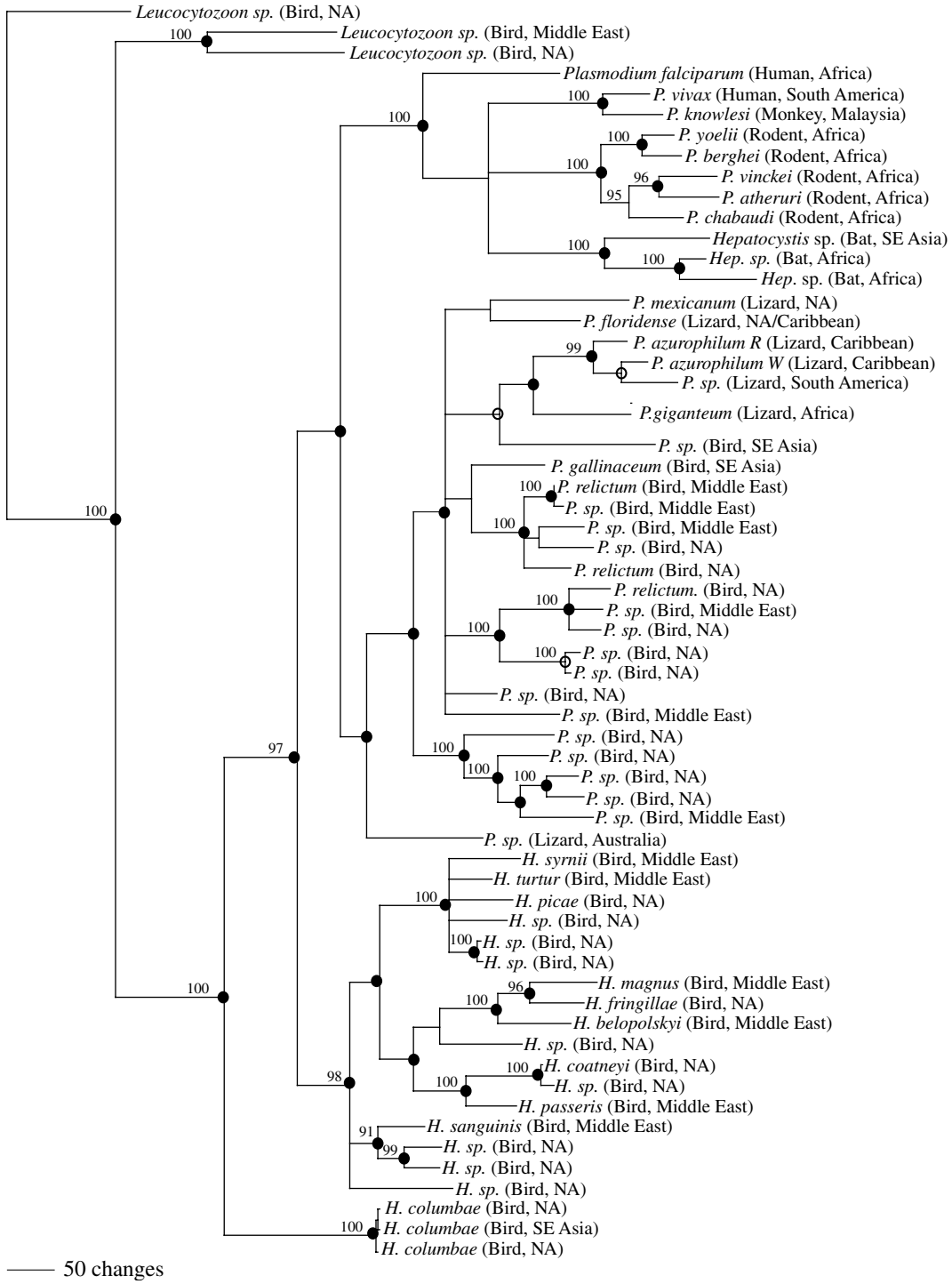

Fig. 2. Majority-rule consensus phylogram recovered using maximum parsimony and two Bayesian analyses of four genes (total 2334 nucleotides) across the parasites' three genomes. Only bipartitions with frequency of observation (posterior probability value) greater than 90% are shown; otherwise, taxa are shown as a polytomy. Dots on nodes indicate nodal support values as estimated using Bayesian analysis ($\geqslant$95% posterior probability indicated by a hollow dot, $\geqslant$99% posterior probability value indicated by a filled dot). Parsimony bootstrap values greater than or equal to 90% are shown above branches. Taxon labels are the genus based on morphology seen in microscope blood smears, and species when that identification could be made with high confidence. Also given are the vertebrate host and geographic region of the samples (NA, North America).

moproteus as proposed by Bennett et al. (1965). These two clades, clearly very distinct, have similar morphology in the vertebrate blood, but develop strikingly different oocysts in the insect hosts (Valkiunas, 2005).

4.3. The enigma of Hepatocystis

The taxonomic status of *Hepatocystis* has been highly unstable through the years. First classified as "*Haema-*

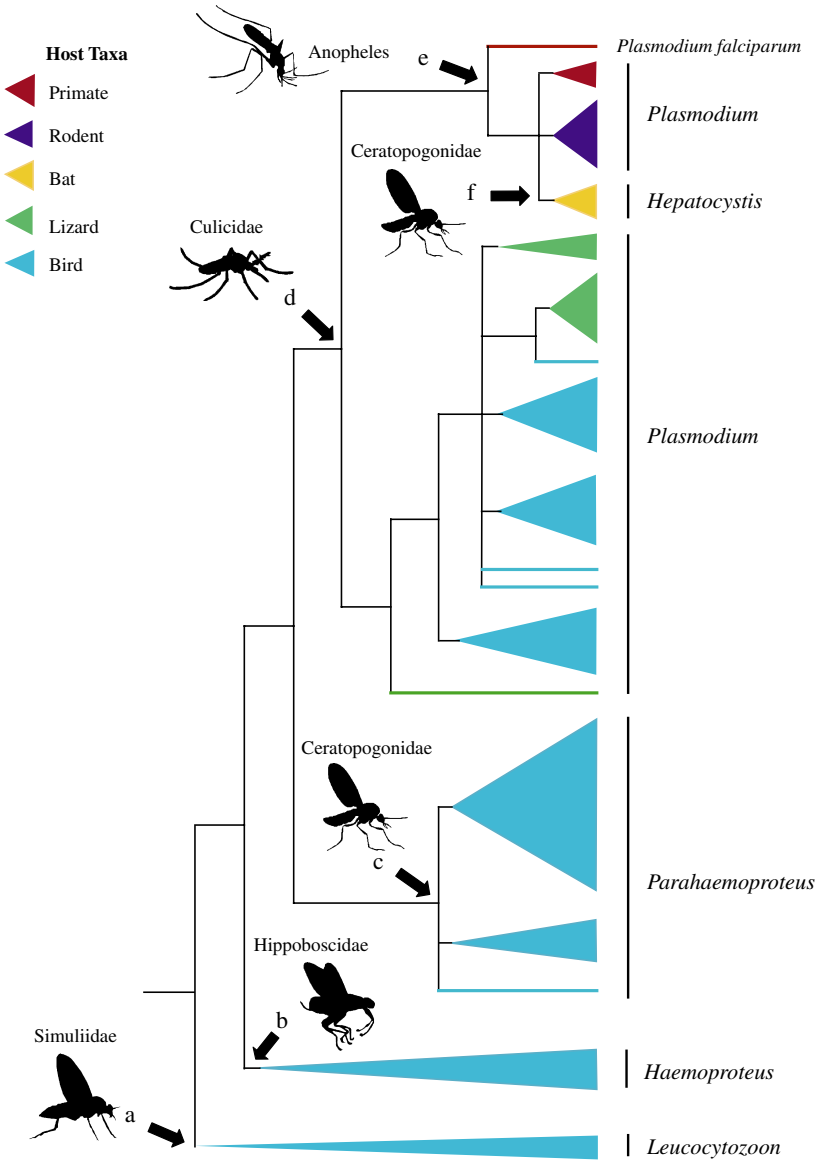

Fig. 3. Summary of the full dataset and analyses for five genera of haemosporidian parasites (*Plasmodium* and relatives) showing relative number of parasite taxa included in the study for each clade (size of triangles). All nodes shown are well supported (⩾90% Bayesian posterior probability values). Given are the vertebrate and insect hosts, and genus and current subgenus names for the parasites. Arrows indicate major vector shift events: (a) The genus *Leucocytozoon* is vectored by blackflies of the family Simuliidae and infects a wide diversity of birds. (b,c) The avian genus *Haemoproteus* is polyphyletic and labeled are its two current subgenera, *Haemoproteus* and *Parahaemoproteus*. The type species, *H. columbae*, is vectored by louse flies (Hippoboscidae) (b) and is a clade of parasites that infect doves world-wide. The greatest diversity of the *Haemoproteus* parasites in birds is of the subgenus *Parahaemoproteus* and is transmitted by biting midges of the family Ceratopognidae (c). This subgenus is a distinct and divergent clade and is most likely its own genus. (d) A well-supported clade contains all species of *Plasmodium* vectored by mosquitoes. A specialization into mosquitoes of the genus Anopheles (e) corresponds with the expansion of *Plasmodium* parasites into mammals including humans. The lineage representing the virulent human malaria parasite, *Plasmodium falciparum*, is labeled at the top of the phylogeny. *Plasmodium*, however, is paraphyletic because it contains parasites of the genus *Hepatocystis* (f). Not shown is the single known *Plasmodium* species that is vectored by a sandfly, rather than a mosquito.

moeba" by Laveran (1899), as all malaria parasites were at the time, it was subsequently transferred to *Plasmodium* (though it is possible that this reclassification was the result of a mixed infection with *Plasmodium gonderi*), and then to *Haemoproteus* when parasitologists could find no evidence for asexual replication in the blood (Garnham, 1951, 1966). The most distinctive characteristic of these parasites is the enormous schizonts (visible to the naked eye) in the liver that produce thousands of daughter cells; Garnham (1948) therefore removed such parasites to their own genus. Searches for the vectors of these parasites were often futile, but all evidence to date has pointed to *Culicoides* midges (Ceratopogonidae) as the likely candidates (Garnham, 1951, 1966). The infections used in our study all came from bats, including one collected in Singapore and two from West Africa and all three isolates form a monophyletic clade that is contained within the mammal *Plasmodium* parasites. One possible explanation for the placement of

Hepatocystis is introgression of the organellar genomes from *Plasmodium* to the ancestor of the sampled *Hepatocystis* species. A phylogeny based on only the single nuclear gene provided little resolution of any clades other than a few closely related species pairs, but did not conflict with the placement of *Hepatocystis* as paraphyletic with respect to *Plasmodium* (data not shown). Other nuclear loci will be necessary to test whether *Hepatocystis* represents an unusual life-history strategy switch from more traditional *Plasmodium* species or if instead, it is an amalgamation of *Plasmodium* organellar DNA (mitochondrial and plastid) and another group.

4.4. Vector switches and the diversification of parasites

Although life-history traits such as storage of malaria pigment and schizogony in the blood are important evolutionary characters, the major events in the evolutionary history of the parasites now appear to have been switches in the insect vector used. Each major clade of parasite is associated with a unique vector family (Fig. 3). The ancestral form of the parasite groups included here was most likely an avian parasite similar to *Leucocytozoon* and transmitted by blackflies (Simulidae). *Haemoproteus* and *Parahaemoproteus* originated with vector shifts from blackflies, first to hippoboscid flies (*Haemoproteus*), and then to biting midges, Ceratopogonidae (*Parahaemoproteus*). The shift from hippoboscid flies to biting midges was concomitant with alterations in development within the vector but no major change in the morphology of the parasites as seen in the blood cells (Valkiunas, 2005). Although both *Parahaemoproteus* and *Haemoproteus* have cosmopolitan distributions, *Parahaemoproteus* parasites have a much wider host range and greater diversity than *Haemoproteus* and infect birds of most recognized orders (Valkiunas, 2005). This suggests that the switch into Ceratopogonids was the prelude to systematic radiation of *Parahaemoproteus* into a broad range of avian hosts worldwide. The ecology and biting behavior of blackflies, hippoboscid flies, and biting midges differ substantially. Most divergent are the hippoboscids, with their long lifespan, virtually flightless adult stages, and movement between hosts primarily by crawling (Bennett et al., 1965). The different environments within new vectors must have presented major challenges for the parasites, and we suspect that the similar morphology of *Haemoproteus* and *Parahaemoproteus* may mask substantial physiological (and genomic) differentiation.

A major life history change occurred with the origin of *Plasmodium*. In addition to an initial round of asexual replication in fixed tissues (in common with the other parasite clades), the *Plasmodium* life cycle includes additional rounds of asexual reproduction (schizogony) in blood cells. There was also a shift to using mosquito vectors, which appears to have led to the exploitation of a greater variety of vertebrate taxa as hosts. Our phylogeny (Figs. 2, 3), in agreement with others (Escalante et al., 1998; Perkins and Schall, 2002) shows that *Plasmodium* entered mammals only once, coincident with a switch from Culicine to Anopheline mosquitoes. A previous study (Escalante and Ayala, 1994) also suggested vector host switches drive the origin of clades as indicated by divergence times of parasites and their vector hosts.

All known *Plasmodium* species use mosquitoes as insect hosts (Culicidae) with the exception of a single species of lizard malaria (*P. mexicanum*), which is transmitted by sandflies (Psychodidae; Ayala and Lee, 1970; Fialho and Schall, 1995). The *Plasmodium* species of birds exploit mosquitoes from the genera *Culex*, *Aedes*, and *Culiseta*, with a few species found in *Anopheles, Psorophora*, and *Mansonia* (Valkiunas, 2005). In contrast, all known vectors of *Plasmodium* of mammals are *Anopheles* (Coatney et al., 1971; Killick-Kendrick, 1978), indicating that the shift of *Plasmodium* into mammals was associated with specialization on anopheline vectors. Why the switch to mammal hosts was associated with specialization on anopheline vectors remains an open and perplexing issue. Thus, the success of malaria parasites in invading bird, lizard, and mammalian vertebrate taxa (a trait unique to *Plasmodium* in relation to other haemosporidian genera) and its systematic diversity may have been driven by use of mosquitoes as vectors.

4.5. The origin of Plasmodium falciparum and the role of host switching

Plasmodium falciparum, the most important *Plasmodium* for human public health, is unusual because of its severe pathology, sequestration of schizont stages by their adherence to capillary walls, and lack of either recrudescence or relapse (Berendt et al., 1990). An early molecular phylogeny of *Plasmodium* found that *P. falciparum* clustered with two avian parasites rather than with those infecting mammals, thus suggesting *P. falciparum* switched to human hosts by lateral transfer from birds at the origin of agriculture (Waters et al., 1991; see also Qari et al., 1996). This result proved contentious (Brooks and McLennan, 1992; Siddall and Barta, 1992) because of the small number of ingroup taxa included, the use of an outgroup belonging to an unrelated phylum, improper rooting, and the use of the 18S rRNA gene which can give spurious results in systematic studies of apicomplexan taxa (see below). Subsequent studies found that the closest identified sister taxon to *P. falciparum* is *P. reichenowi* of chimpanzee hosts, and that the bird and mammal parasites did not cluster (Escalante and Ayala, 1994; Escalante et al., 1995, 1998; Perkins and Schall, 2002). Although the origin of *P. falciparum* from lateral transfer of an avian malaria parasite should now be discounted, both popular (Diamond, 1998) and technical (Wolfe et al., 2007; Hagner et al., 2007) accounts continue to revisit the issue. The new four-gene phylogeny places *P. falciparum* solidly (99% PP) within the clade of other mammalian malaria parasites and decisively refutes a close relationship with the avian

parasites. The move into mammals by *Plasmodium* thus occurred just once in evolutionary history.

Host switching does appear to have occurred within the two broad groups of *Plasmodium*, however. Within the mammalian parasite clade, others have shown, with denser taxon sampling, that host switching between primates (including humans) appears to have repeatedly taken place (Escalante and Ayala, 1994; Cornejo and Escalante, 2006; Mu et al., 2005). Within the clade infecting birds and reptiles, we show here that *Plasmodium* has shifted between birds and lizards several times. Because the erythrocytes of bird and squamate reptiles are nucleated, this may have facilitated multiple shifts between vertebrate hosts that otherwise appear very different in physiology and ecology.

4.6. The future of malaria parasite systematics

In addition to their profound effects on human and wildlife populations and the enormous body of research that has gone into developing vaccines and treatments for the disease, the malaria parasites have also been widely used as model systems for studies in a great range of issues in ecology and evolution. All of these efforts, however, depend on a solid phylogenetic framework. For example, an appropriate model for studies of *P. falciparum* in non-human hosts appears to be *P. reichenowi*, although molecular data are available only for a single sample collected from an infected chimpanzee. No other known *Plasmodium* species is closely related to *P. falciparum* (Fig. 2), with a long branch between the *P. falciparum–P. reichenowi* clade and the other *Plasmodium* of mammals (Perkins and Schall, 2002).

Unfortunately, the systematic study of malaria parasites has been limited to a very small set of loci, and our phylogeny is the first to combine data for more than a single gene for more than just *Plasmodium* taxa and from a diversity of vertebrate host groups. Previous multi-gene phylogenetic studies have focused primarily on the *Plasmodium* taxa of primates (Escalante et al., 1995; Mu et al., 2005) or rodents (Perkins et al., 2007). Locating suitable loci has been difficult. The rRNA genes, widely used in phylogenetic analyses (Hillis and Dixon, 1991), are highly problematic in the haemosporidians. The 18S rRNA gene copies do not exhibit concerted evolution in malaria parasites, but exist as several independently evolving, paralogous loci (Corredor and Enea, 1993; Li et al., 1997; Rogers et al., 1995). The copies of this gene are expressed during different points in the life cycle, and have substantially diverged (Rogers et al., 1995). Even copies expressed during a single point in the life cycle may not be homologous across taxa if the stage of expression of the copies has switched over the evolutionary history of the parasites. These issues may account for the difficulty in obtaining reliable alignments for the 18S rRNA gene in apicomplexans (Morrison and Ellis, 1997). Surface protein molecules have been used for some studies (Escalante et al., 1995; McCutchan et al., 1996), but these genes, which code for the proteins that interact with the host are under strong selection (Hughes and Hughes, 1995). Mitochondrial genes, the other common choices for phylogenetic work (Harrison, 1989; Simon, 1991) are limited to the three that remain in the mitochondrial genome of apicomplexans (Feagin, 2000). In the future, loci from conserved housekeeping genes need to be included, though with caution because many genes are duplicated in the *Plasmodium* genome (Kooij et al., 2005). The most important step, we would argue, to resolve these relationships, is going to be adding many more taxa, particularly those that have been placed in other genera or subgenera based on morphological or life-history deviations from *Plasmodium*. These include the less speciose genera that infect squamate reptiles (*Haemocystidium* and *Saurocytozoon*) and Old World mammals (*Nycteria*, *Polychromophilus*, and *Rayella*), which may well represent the "lost" ancestors of the lineage that gave rise to *P. falciparum*.

4.7. What is a "malaria parasite"?

The term "malaria parasite" is in wide use in the technical literature, so the term should be used with precision. One suggestion is that "malaria parasite" be restricted to species with asexual replication in the vertebrate blood (*Plasmodium*) (Valkiunas et al., 2005). However, common names should have phylogenetic significance. If the *Plasmodium* clade represents malaria parasites, then *Hepatocystis*, with very different morphology and life cycle, must be included, and parasites much more similar to *Plasmodium* in life cycle and morphology (*Haemoproteus* and *Parahaemoproteus*) would not. We propose that this awkward situation is best resolved by including the monophyletic clade that includes *Haemoproteus* + *Parahaemoproteus* + *Hepatocystis* + *Plasmodium* under the rubric of malaria parasites.

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