Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes

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SUMMARY

Malaria parasites in the genus *Plasmodium* are now placed within 11 subgenera based on morphology under the light microscope, life-history traits, and host taxon. The phylogenetic significance of these characters, however, is problematic because the observed variation could be homoplasious. Using *Plasmodium* infections found in 2632 birds of many avian families collected in the USA, and several samples from other locations, we compared identifications to subgenus based on morphology in blood smears with a 2-gene molecular phylogeny (the first for avian *Plasmodium*) to determine if the 5 avian *Plasmodium* subgenera represent monophyletic groups. Phylogenetic trees recovered by parsimony, likelihood, and Bayesian methods presented nearly identical topologies. The analysis allowed testing the hypothesis of monophyly for the subgenera. Monophyly of the subgenera *Haemamoeba*, *Huffia*, and *Bennettinia* was supported by the analysis. The distinctive morphology of *Haemamoeba* species appears to have evolved once. Most samples identified to *Novyella* also fell within a monophyletic clade with the exception of 2 samples that fell basal to all other avian *Plasmodium*. Samples of the subgenus *Giovannolaia* did not form a monophyletic group. Thus, the characters used by parasitologists for over a century to define subgenera of *Plasmodium* vary in their phylogenetic significance.

Key words: *Plasmodium*, avian malaria parasites, cytochrome *b*, cytochrome oxidase I, subgenera, monophyly.

INTRODUCTION

The discovery of a malaria parasite in human blood by Laveran in 1880 was followed by recognition that a substantial diversity of related parasite species is found in reptile, bird, and mammal hosts almost worldwide (reviewed by Garnham, 1966). Debate quickly emerged on how to place these species into genera, as well as over the relationships of species within each genus. The central issue for over a century has been the choice of appropriate characters to define taxa of the parasites from species to families (Corradetti *et al*. 1963; Garnham, 1966; Valkiunas, 2005). Modern systematic biology requires that higher taxa represent monophyletic groups of species, and the characters used to define such taxa must be phylogenetically informative. That is, taxa must be defined based on shared derived character states (synapomorphies). The characters traditionally used to define subgenera of *Plasmodium*, for example, include morphology seen under the light microscope, details of life-cycles, insect and vertebrate host range and, more recently, ultrastructural morphology (Garnham, 1966; Laird, 1998; Valkiunas, 2005). However, the phylogenetic significance of these characters has long been problematic (Corradetti *et al*. 1963; Garnham, 1966; Manwell, 1936) and recent molecular studies have recalled the issue (Escalante *et al*. 1998; Perkins and Schall, 2002).

Based on traditional characters, fully 11 subgenera are now considered valid for *Plasmodium*, with a new subgenus being erected only within the past decade (Garnham, 1966; Valkiunas, 1997). A recent analysis of gene sequences (the cytochrome *b* gene from the mitochondrial genome) supported the monophyly of each of the 3 named subgenera of *Plasmodium* found in mammal hosts (*Laverania* of humans/chimpanzees, *Plasmodium* in other apes, humans, and monkeys, and *Vinckeia* in rodents) (Perkins and Schall, 2002; Perkins *et al*. 2006). However, the status of the primary diversity of *Plasmodium*, which includes the species exploiting reptiles and birds as the vertebrate hosts, was unresolved. Kissinger *et al*. (2002) used sequences of the small-subunit ribosomal RNA gene to examine relationships of 5 *Plasmodium* species from avian hosts and concluded that the currently accepted subgenera of avian malaria parasites are not phylogenetically valid (the subgenera do not represent independent evolutionary groups of species). Molecular phylogenies also cast doubt on the value of traditional characters used to define higher taxa of

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malaria parasites (Escalante *et al*. 1998; Perkins and Schall, 2002). That is, characters such as blood cell type exploited, shape and size of schizonts and gametocytes in the blood, periodicity of infection in vertebrate host, and number of merozoites in a mature schizont may often be homoplasious (character states evolved independently in different lineages). In contrast, a detailed study of several species concepts (morphology, genetic, and phylogenetic) for an assemblage of avian haemosporidian parasites revealed that morphology alone usually assigns parasite lineages to monophyletic groups that may represent species (Martinsen *et al*. 2006).

We ask here if the recognized subgenera of avian *Plasmodium*, erected primarily on morphology, represent monophyletic groupings of species. We use sequences for 2 genes from the parasite's mitochondrial genome, cytochrome *b* and cytochrome oxidase I, to recover a phylogeny of *Plasmodium* identified to subgenera based on morphology seen in blood smears. The infections were identified from a large sample of birds collected primarily from Vermont and California, USA. This is the first 2-gene phylogenetic study of avian *Plasmodium*. Our goal is not to propose a taxonomic revision of the genus *Plasmodium*, but to determine if the often-striking morphological characteristics used to define the subgenera are phylogenetically significant. If these characters do not successfully define monophyletic groups, this would suggest they are under differing selective pressure within parasite evolutionary groups, an intriguing and significant finding. The results thus cast light on the evolutionary significance of morphological characters used for more than a century to define groups of *Plasmodium* species.

MATERIALS AND METHODS

Birds were collected from sites throughout Vermont (USA) and a site in northern California (USA) near the town of Hopland in Mendocino county (University of California Hopland Research and Extension Center) using mist-nets and potter traps. After each bird was identified, a blood sample was obtained by puncture of the brachial vein and used to produce a thin smear to be fixed in absolute methanol and blood dots dried on filter paper to be stored frozen with silica gel beads. All sampling was conducted under governmental permits and University of Vermont animal care guidelines. Several other samples were provided by colleagues, 1 from Uganda that provided sufficient material for proper diagnosis, and 2 samples from Israel with highly confident identification (Martinsen *et al*. 2006). Sequence data for the 2 genes from *P. relictum* of Hawaiian birds were extracted from GenBank (AY733090). This is a very well-characterized sample of *P. relictum* and thus identification to species is reliably corroborated (Beadell and Fleischer, 2005). The subgenus *Bennettinia* presently is known to include only *P. juxtanucleare* of domestic chickens (Valkiunas, 1997), and is represented here by 3 samples collected in Vietnam. These samples are confidently assigned to *P. juxtanucleare* because they are well characterized by researchers at the Pasteur Institute. Data from GenBank for another isolate of *P. juxtanucleare* from Japan are also included in the analysis (YP717012).

Smears were treated with Giemsa stain (1 : 10, pH 7·0, 50 min) and the entirety of each slide examined at 1000 × magnification. Only infections containing substantial numbers of schizonts and gametocytes were used to determine subgenus. Most *Plasmodium* infections revealed parasitaemia too low for confident diagnosis to subgenus, and were discarded from the study. Morphological characters used to identify the subgenera are presented in Table 1. Only characters apparent on the stained blood smears were available for the study. Although some of these characters overlap between subgenera, a combination allowed confident identification.

DNA was extracted from dried blood dots with the DNeasy kit (Qiagen, USA). A final 614 bp segment of the cytochrome *b* gene (cyt *b*) and a 982 bp segment of the cytochrome oxidase I gene (coI) were amplified using nested PCR. Outer reactions for cyt *b* gene used primers DW2 (5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′) and DW4 (5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′) (Escalante *et al*. 1998; Perkins and Schall, 2002). Outer reactions for the coI gene used primers CoIoutF (5′-CTA TTT ATG GTT TTC ATT TT ATT TGG TA-3′) and CoIoutR (5′-AGG AAT ACT AGG CAT TAC ATT AAA TCC-3′) (Perkins *et al*. 2006). Both outer reactions contained 2 μl of extraction product, 1 μl of each 10 μM primer, and a Ready-to-Go PCR bead (Amersham, USA). An initial denaturation for 4 min at 94 °C was followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 68 °C for 50 sec. The inner reactions contained 1 μl of the outer product and used primers DW1 (5′-CAT ATC CTA AAG GAT TAG AGC TAC CTT GTA A-3′) and DW3 (5′-TGC TGT ATC ATA CCC TAA AG-3′) (Perkins and Schall, 2002) for cyt *b*, and CoIinF (5′-ATG ATA TTT ACA RTT CAYGGW ATT ATT ATG-3″) and CoIinR (5′-GTA TTT TCT CGT AAT GTT TTA CCA AAG AA-3′) (Perkins *et al*. 2006) for coI. PCR conditions for inner reactions were an initial step of 94 °C for 1 min followed by 40 cycles of 94 °C for 20 sec, 52 °C for 20 sec, and 68 °C for 30 sec, and a final step of 7 min at 68 °C. A negative control was used for each PCR run, and no contamination was detected by gel electrophoresis of the PCR products.

After purification (ExoSAP-It, USB, USA), the PCR products were sequenced using BigDye Terminator v3.0 and analysed on an ABI Genetic Analyzer (ABI, USA) at the University of Vermont

Table 1. Currently recognized subgenera of *Plasmodium* from avian hosts with key defining characters seen in stained blood smears

(Example species for each subgenus are also provided. Diagnostic characters were extracted from the type description for each subgenus, Valkiunas (2005), Garnham (1966), and Laird (1998).)

Subgenus	Schizont characteristics	Gametocyte characteristics	Example species
Haemamoeba (Grassi and Feletti, 1890)	Mature schizonts large and exceed the size of the host cell nucleus and also commonly displace the host cell nucleus	Mature gametocytes round, oval, or irregular in form and substantially exceeds the size of the host cell nucleus	*P. relictum*, *P. cathemerium*, *P. matutinum*, *P. gallinaceum*
Huffia (Corradetti, Garnham and Laird, 1963)	Mature schizonts variable in form and size, contain plentiful cytoplasm, and are commonly found in immature red blood cells	Gametocytes are elongated	*P. elongatum*, *P. hermani*
Giovannolaia (Corradetti, Garnham and Laird, 1963)	Schizonts are substantially larger than host cell nucleus, contain plentiful cytoplasm, and are not present in immature red blood cells	Gametocytes are elongated	*P. circumflexum*, *P. polare*, *P. lophurae*
Novyella (Corradetti, Garnham and Laird, 1963)	Schizonts are not larger or only slighty larger than host cell nucleus and contain scanty cytoplasm	Gametocytes are elongated	*P. vaughani*, *P. rouxi*, *P. hexamerium*, *P. nucleophilum*
Bennettinia (Valkiunas, 1997)	Schizonts are often round, do not exceed the size of the host cell nucleus, contain scant cytoplasm, and stick to the host cell nucleus	Gametocytes vary in shape but are generally round or oval, do not exceed the size of the host cell nucleus, and stick to that nucleus	Only 1 known, *P. juxtanucleare*

Cancer Center. The cytochrome *b* gene fragment was sequenced using the DW1 primer, and the cytochrome oxidase I gene fragment with the CoIinF primer. Sequences were edited using Sequencher (Genecodes, USA), aligned by eye in PAUP* 4.0b10 (Swofford, 2002), and examined for the presence of stop codons using MacClade version 4.03 (Maddison and Maddison, 2001). Indels were not observed for either gene, so alignment was unambiguous. If inspection of any electropherogram revealed ambiguous base calls, the sample was re-amplified, sequenced, and edited. Continued presence of ambiguous bases suggested a mixed-species infection, and these samples were discarded from the study.

Phylogenetic analysis used 2 rodent malaria parasites, *Plasmodium berghei* and *P. chabaudi* as outgroup taxa because a previous study (Perkins and Schall, 2002) and our confirming analysis demonstrated that these species are closely related to but not contained within the avian *Plasmodium* clade. Sequence data for the rodent parasites were obtained using the same protocols described above for samples provided by R. Carter (University of Edinburgh). Phylogenetic reconstruction was conducted under both parsimony and likelihood frameworks and hypothesis testing was carried out using the Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999).

Both unweighted and weighted parsimony analyses were performed. Unweighted maximum parsimony analysis involved 10 000 random stepwise-addition heuristic replicates as implemented in PAUP* 4.0b10 (Swofford, 2002). A site-specific rate weighting scheme was also employed, a method shown to be useful when gene conservatism, unequal nucleotide frequencies, and differences in substitution rates are encountered among and within datasets, such as for the cyt *b* and coI genes used in our phylogenetic analysis (data not shown) (Kjer *et al*. 2001). Each character from the 2-gene dataset was assigned to one of 5 rate classes based upon a fast heuristic bootstrap analysis (1000 replicates) in PAUP* (Kjer *et al*. 2001). A heuristic search with 10 000 random stepwise-addition replicates was then performed in PAUP* with each character weighted inversely to its rate of change. Nodal support values were generated using 10 000 heuristic bootstrap replications under both the unweighted and site-specific rate weighting schemes.

Phylogenetic analysis using likelihood criteria was conducted using the Markov Chain Monte Carlo (MCMC) sampling regime in MrBayes (MrBayes v3.0b4; Hulsenbeck and Ronquist, 2001). The most appropriate model of evolution was selected separately for each gene using the Akaike Information Criterion (ModelTest version 3.06; Posada and Crandall, 1998). A general time-reversible model with a gamma distribution for variable sites and proportion of sites as invariable (GTR + Γ + I) was

chosen for both cyt *b* and coI. Starting parameters used for the Bayesian analysis included a starting tree estimated from neighbour-joining methods in PAUP*. Additionally, the data were partitioned into the 2 genes, with a separate model of evolution (GTR + Γ + I) designated for each data partition. The MCMC sampling regime was run twice independently for 1 million generations with sampling every 100 generations (MrBayes v3.0b4). A total of 10 001 trees resulted from each analysis, with the first 1000 trees discarded as burn-in or suboptimal trees. The 9001 remaining trees from each analysis were then combined ($N = 18\,002$) and used to calculate posterior probability values.

To compare the topology resulting from our analyses to the hypothesis that the subgenera of avian *Plasmodium* are monophyletic, hypothesis testing was carried out using maximum likelihood analysis and the Shimodaira and Hasegawa test in PAUP*. A GTR + Γ + I model of evolution was appropriated to the combined gene dataset using the Akaike Information Criterion of ModelTest v3.06 (Posada and Crandall, 1998) and a likelihood heuristic search involving 100 random stepwise-addition replicates performed. The resulting maximum likelihood tree was then used to construct an alternative topology by branch rearrangement in MacClade, such that each subgenus was constrained to a clade (and thus to monophyly). This alternative topology was then loaded as a constraint for a maximum likelihood analysis identical to that above. In this additional likelihood analysis, only trees in which the subgenera were monophyletic were evaluated. The best tree obtained from the constrained maximum likelihood analysis was then compared to the observed maximum likelihood tree using the Shimodaira and Hasegawa test including 1000 bootstrap replicates with RELL approximation in PAUP* (Shimodaira and Hasegawa, 1999).

RESULTS

A total of 2200 birds from Vermont and 432 birds from California were screened by light microscopy for malaria parasites, with 204 (Vermont) and 6 (California) found infected with *Plasmodium*. Most of these infections ($N = 180$) were low-grade parasitaemia and/or lacked one of the developmental stages, and so were discarded from the study. In addition, 5 infections presented ambiguous sequences due to mixed infections of the genera *Plasmodium* and *Haemoproteus*, and these samples were discarded (because genus-specific primers have not yet been developed for the coI gene). This left 25 infections from Vermont and California from a range of passerine families that could be confidently placed into subgenera. Addition of 2 samples from Israel, 1 from Uganda, 3 from Vietnam and the GenBank sequences available for the Hawaiian *P. relictum* and a Japanese isolate of *P. juxtanucleare* resulted in a total of 33 avian *Plasmodium* samples.

Three samples were identified to the subgenus *Huffia*, 9 samples to the subgenus *Haemamoeba*, 3 samples to the subgenus *Giovannolaia*, 4 samples to the subgenus *Bennettinia*, and 14 samples to the subgenus *Novyella*. For each of these samples and the 2 outgroup rodent *Plasmodium* taxa, a total of 1596 nucleotides from the 2 genes were included in the analysis; sequences generated in this study are deposited in GenBank (Accession numbers EF011166–EF011231).

The unweighted maximum parsimony heuristic search resulted in 6 equally parsimonious trees that differed only in the placement of 2 *Novyella* samples within the major *Novyella* clade and the order of appearance of identical haplotypes within clades. The weighted parsimony analysis resulted in 3 equally parsimonious trees and a consensus tree identical to that estimated by the unweighted analysis. The strict consensus phylogram summarizing the results from the weighted parsimony analysis including bootstrap support values, is presented in Fig. 1. Bayesian MCMC analysis estimated an almost identical topology, with only slight rearrangement of 2 *Novyella* samples within the major *Novyella* clade. Posterior probability values from the Bayesian MCMC analysis are provided in Fig. 1. The likelihood analysis resulted in a topology that differed only in the placement of 1 *Novyella* sample within the major *Novyella* clade.

The 3 samples identified to the subgenus *Huffia* had identical sequence data for both genes. These were found in a House Finch (*Carpodacus mexicanus*) and 2 Song Sparrows (*Melospiza melodia*), all sampled in Vermont. These samples fell onto a well-supported long branch, and thus likely represent a distinct clade. All samples identified to the subgenus *Haemamoeba* fell into a well-supported clade, with bootstrap support and posterior probability values of 96 and 99% respectively. This *Haemamoeba* clade includes 2 samples identified as *P. relictum*, 1 from Israel and 1 from Hawaii. Most samples identified to the subgenus *Novyella* fell into a well-supported clade according to Bayesian analysis, including samples from Vermont and California. Two other samples, with sequences differing by only a single base pair, were identified as the subgenus *Novyella* from 2 Red-winged Blackbirds (*Agelaius phoeniceus*) and fell onto a long well-supported branch basal to all other avian *Plasmodium* in the study. The 3 samples of *Bennettinia* from Vietnam produced identical sequences and differed by only a single base pair from the Japanese sample extracted from GenBank. These samples fell on a long branch basal to most other *Plasmodium* included in the study. Thus, the morphologically distinctive subgenus *Bennettinia* appears to form a valid clade. Although only 3 samples were identified to the subgenus *Giovannolaia*, these

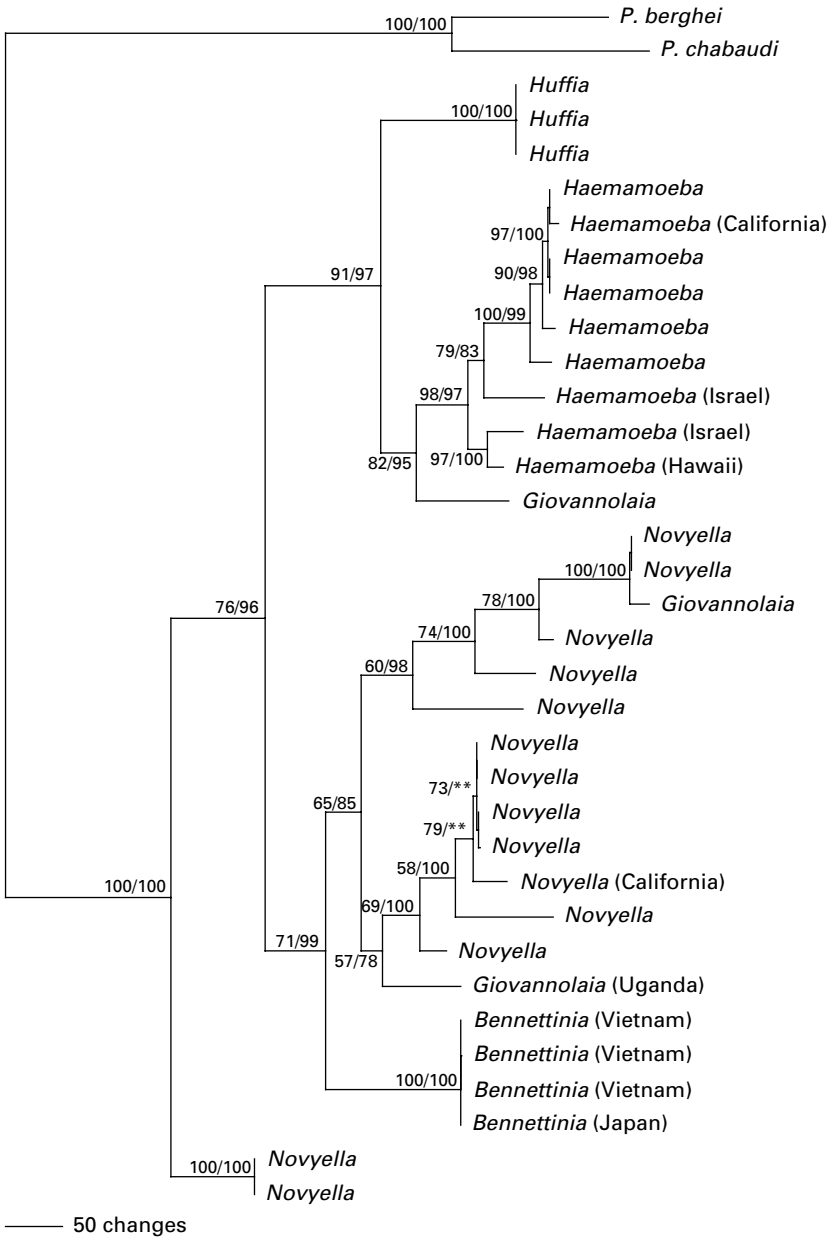

Fig. 1. Phylogenetic relationships of the avian subgenera of *Plasmodium* based upon 614 nucleotides of the cytochrome *b* gene and 982 nucleotides of the cytochrome oxidase I gene. A weighted maximum parsimony consensus phylogram ($N=3$ trees) is presented, with bootstrap values followed by posterior probabilities for each node. Posterior probability values are not provided for nodes recovered only by maximum parsimony analysis as indicated by asterisks. Locations for samples collected outside of Vermont, USA, are provided. Outgroup taxa include the rodent malaria parasites, *P. berghei* and *P. chabaudi*.

fell within 3 distant clades on the phylogeny, and clearly did not form a monophyletic group. One from Vermont (from a Northern Waterthrush, the warbler *Seiurus noveboracensis*) fell as a sister taxon to the *Haemamoeba* clade, and the other 2 fell within a large clade that contained most of the '*Novyella*' samples (from an American Robin, *Turdus migratorius*, from Vermont, and from the Ugandan African Fish Eagle, *Haliaeetus vocifer*).

The subgenera *Huffia*, *Haemamoeba*, and *Bennettinia* are monophyletic by all 3 methods of phylogenetic inference. As representatives of the subgenera *Novyella* and *Giovannolaia* were interspersed only with each other and not contained within nodes defining the subgenera *Huffia*, *Haemamoeba*, and/or *Bennettinia*, a single constraint tree was constructed in which both of these subgenera were monophyletic. The hypothesis of monophyly for *Novyella* and *Giovannolaia* was then tested against the observed topology using the Shimodaira and Hasegawa test. Results of this test indicate that the constraint topology was significantly different than the observed topology by likelihood criteria ($P<0{\cdot}0001$). Thus, the constraint topology

displaying monophyly for the subgenera *Novyella* and *Giovannolaia* was seen as a significantly worse topology according to likelihood analysis of our 2-gene mitochondrial dataset.

DISCUSSION

Early efforts to understand the diversity of malaria parasites centred on defining the evolutionary relationships of the parasites using morphological characters from stages found in both the vertebrate and insect host. Also considered important were life-history features (variation in details of the life-cycle) and the taxon of both vertebrate and insect host (reviewed by Garnham, 1966; Valkiunas, 2005). The phylogenetic significance of these characters was questioned long ago by Manwell (1936) when he proposed that only genetic data would reveal the true relationships of the parasites. When Corradetti *et al*. (1963) reviewed the avian *Plasmodium* and erected 2 new subgenera (*Novyella* and *Giovannolaia*) they viewed the use of morphological characters as an imperfect guide to the true evolutionary relationships of *Plasmodium* species. The early doubts of Manwell, Garnham, Corradetti and others are now mirrored by findings based on new phylogenies recovered using gene sequence data. These results suggest that life-history traits of the biology of malaria parasites have evolved independently in phylogenetically distinct lineages (Escalante *et al*. 1998; Perkins and Schall, 2002; Perez-Tris *et al*. 2005, but see Valkiunas *et al*. 2005). For example, *P. giganteum*, a parasite of west African lizards, that produces enormous schizonts in erythrocytes containing ~100 merozoites, is not related to other *Plasmodium* giants, but is the closest sister to the relatively tiny *P. agamae* (Perkins and Schall, 2002).

After a large number of *Plasmodium* species were described (now ~180) from mammals (primarily from primates and rodents), birds (from a very broad range of families), and lizards (also from many families), parasitologists erected an array of subgenera in an attempt to maintain the genus *Plasmodium* as a single taxon (Corradetti *et al*. 1963; Garnham, 1966; Valkiunas, 2005). Previous molecular phylogenies supported the monophyly of the subgenera of *Plasmodium* of mammals, but the relationships of parasites of birds and reptiles remained unresolved (Perkins and Schall, 2002; Beadell *et al*. 2004; Szymanski and Lovette, 2005). Kissinger *et al*. (2002) presented a phylogeny based on an rRNA gene for several isolates of avian malaria identified to subgenus, and questioned the value of the subgenera erected for the bird *Plasmodium*. Our results are less pessimistic than those of Kissinger *et al*. (2002) because the new analysis supports 3 of the 5 named subgenera of avian *Plasmodium* (below). We approached the problem by including a larger sample of infections, using sequence data from 2 genes, and analysing the data with a spectrum of methods, including hypothesis testing.

A major challenge encountered in our study was logistical – finding infections with sufficient parasites of all stages needed to score the morphological characters that define the subgenera. Only 14% of the *Plasmodium* infections contained a full range of immature and mature schizonts and gametocytes that were required for comparison to literature descriptions of the parasites. Valkiunas (2005) also casts a warning that using only a few parasites or a single life-stage will result in many errors in identification. Therefore, we used only infections with plentiful parasites of all stages, and examined each slide completely at least 3 times. Any other protocol will present questionable results.

The study resulted in a phylogeny that was well resolved and with very similar topology by all methods of analysis. This phylogeny suggests a new hypothesis for the relationships between the parasites and offers some insight into the venerable issue of the value of morphology in systematic studies of *Plasmodium*. Five clades are apparent. One clade included 3 samples, apparently from the same *Plasmodium* species, with the *Huffia* morphology of variable schizont size and shape, plentiful cytoplasm, elongated gametocytes, and immature erythrocytes as host cells. Concluding that *Huffia* represents a monophyletic group is premature, but the samples fell onto a well-supported long branch as would be expected if *Huffia* is indeed a distinct clade. A second clade included parasites matching the morphology of *Haemamoeba*, with round or oval schizonts and gametocytes that often displace the host cell nucleus. Within this clade were samples identified definitively as *P. relictum*, 1 from Israel and 1 from Hawaii. *P. relictum* has been implicated in the catastrophic decline of the endemic Hawaiian avifauna (van Riper *et al*. 1986; Atkinson and van Riper, 1991). The *P. relictum* from Hawaii falls closely with the sample identified as *P. relictum* from Israel. The third clade includes most samples identified to *Novyella* and some *Giovannolaia*, and contains species with diverse morphology, but always elongated gametocytes. The morphological distinctions between *Novyella* and *Giovannolaia* are not clear, and have been viewed as ambiguous (Corradetti *et al*. 1963; Seed and Manwell, 1977; Laird, 1998). Indeed, upon erecting the subgenera *Novyella* and *Giovannolaia*, Corradetti *et al*. (1963) admitted a difficulty in assignment of some parasite species to one of these 2 subgenera. Our results clearly demonstrate that these subgenera do not form 2 monophyletic clades, and the morphological characters differentiating the 2 subgenera do not appear to be phylogenetically informative. The fourth clade includes all 4 samples identified to the monospecific subgenus *Bennettinia*, and this clade fell basal to almost all other *Plasmodium* in the study. *Bennettinia* was erected by Valkiunas (1997)

for *P. juxtanucleare*, a distinctive small parasite that usually adheres to the host cell nucleus. The last clade was identified from only 2 samples with the *Novyella* morphology, but falling basal to all avian *Plasmodium*. An enigmatic single sample, with the morphology of *Giovannolaia*, fell as a sister taxon to the entire subgenus *Haemamoeba*. The currently recognized subgenus *Giovannolaia* appears invalid.

If this phylogenetic hypothesis is correct, it suggests that the characters used for over a century by parasitologists to define relationships among *Plasmodium* taxa are not always informative for systematic studies. However, the adaptive significance of the variation in morphology and life-history traits (such as use of the type of host cell) remains an intriguing open question. For example, the striking morphology of *Haemamoeba* species, including large round gametocytes and prominent host cell nucleus displacement, represents an important variant in how the parasite grows and functions within its host cell, and seems to have evolved once. Similarly, ready occupation of immature red blood cells indicates the unique evolutionary lineage of the *Huffia* samples identified in this study. *P. juxtanucleare* has a morphology similar to the *Haemamoeba* species (round schizonts and gametocytes), but the parasite is very small, and often sticks to the host cell nucleus. Valkiunas (1997) noted the unusual morphology of this species and its very wide geographical distribution in the domestic chicken and its relatives, and erected the subgenus *Bennettinia* to include only *P. juxtanucleare*. His analysis is supported here. The unusual morphology of *P. justanucleare* appears to represent a unique evolutionary event in the evolution of avian malaria parasites. In contrast, the subtle variation in morphology seen as important in distinguishing parasites among the subgenera *Novyella* and *Giovannolaia* appears to be plastic over evolutionary time and does not concord with distinct clades. The character of elongate gametocytes may be biologically important, although its significance to the identification of evolutionarily independent groups needs to be further explored because the subgenera *Huffia*, *Novyella*, and *Giovannolaia* all display this trait and the subgenera *Novyella* and *Giovannolaia* do not represent monophyletic groups as indicated by our analyses. Molecular phylogenies, therefore, can reveal more than simply the relationships of the diversity of *Plasmodium* species, but can also focus attention on the question of adaptive significance for the morphological and life-history differences among species.

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