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Phylogeny of haemosporidian blood parasites revealed by a multi-gene approach ☆

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ABSTRACT

The apicomplexan order Haemosporida is a clade of unicellular blood parasites that infect a variety of reptilian, avian and mammalian hosts. Among them are the agents of human malaria, parasites of the genus *Plasmodium*, which pose a major threat to human health. Illuminating the evolutionary history of Haemosporida may help us in understanding their enormous biological diversity, as well as tracing the multiple host switches and associated acquisitions of novel life-history traits. However, the deep-level phylogenetic relationships among major haemosporidian clades have remained enigmatic because the datasets employed in phylogenetic analyses were severely limited in either gene coverage or taxon sampling. Using a PCR-based approach that employs a novel set of primers, we sequenced fragments of 21 nuclear genes from seven haemosporidian parasites of the genera *Leucocytozoon*, *Haemoproteus*, *Parahaemoproteus*, *Polychromophilus* and *Plasmodium*. After addition of genomic data from 25 apicomplexan species, the unreduced alignment comprised 20,580 bp from 32 species. Phylogenetic analyses were performed based on nucleotide, codon and amino acid data employing Bayesian inference, maximum likelihood and maximum parsimony. All analyses resulted in highly congruent topologies. We found consistent support for a basal position of *Leucocytozoon* within Haemosporida. In contrast to all previous studies, we recovered a sister group relationship between the genera *Polychromophilus* and *Plasmodium*. Within *Plasmodium*, the sauropsid and mammal-infecting lineages were recovered as sister clades. Support for these relationships was high in nearly all trees, revealing a novel phylogeny of Haemosporida, which is robust to the choice of the outgroup and the method of tree inference.

1. Introduction

Haemosporida are protozoan blood parasites with complex life cycles that infect a great variety of vertebrate hosts. Haemosporidians are member of the phylum Apicomplexa and include the genus *Plasmodium*. At least five *Plasmodium* species have independently acquired the ability to infect humans (Escalante et al., 1995; Cox-Singh et al., 2008). As agents of human malaria, *Plasmodium* parasites are one of the greatest threats to human health (WHO, 2013). Surveys of blood parasites in vertebrate wildlife have revealed a rich diversity of haemosporidian lineages infecting reptiles, birds and mammals (e.g., Ricklefs and Fallon, 2002; Duval et al., 2007; Valkiūnas et al., 2008; Bensch et al., 2009; Chasar et al., 2009). However, due to their importance in medical research, most studies have focused on *Plasmodium* species of primates and rodents. Large-scale genome sequencing efforts have produced several complete genomes of these parasites (e.g., Carlton et al., 2002, 2008; Gardner et al., 2002; Hall et al., 2005; Pain et al.,

Abbreviations: *asl*, adenylosuccinate lyase; *clpc*, caseinolytic protease; *coI*, cytochrome oxidase I; *cytb*, cytochrome b; ML, maximum likelihood; MP, maximum parsimony.

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2008; Tachibana et al., 2012; Otto et al., 2014), while other key taxa for the understanding of haemosporidian evolution are only scarcely represented in public databases. Therefore, deep-level phylogenetic relationships among major haemosporidian lineages are still poorly resolved. Yet, our understanding of the emergence of new diseases and the acquisition of novel life-history traits by parasites depends on the knowledge of a solid phylogenetic backbone (Lefevre et al., 2007).

The order Haemosporida currently contains 15 extant genera, though the vast majority of the more than 500 described species have been assigned to the four genera *Plasmodium*, *Hepatocystis*, *Haemoproteus* and *Leucocytozoon*. Members of the genus *Plasmodium* infect a wide range of vertebrate hosts, whereas *Leucocytozoon*, and *Haemoproteus* are limited to sauropsids and *Hepatocystis* is only found in mammals (predominantly bats and primates). Some of the other genera only contain a single described species for which molecular data is not available and the taxonomic status of some genera remains uncertain (see Perkins (2014) for a review of the history of haemosporidian systematics), e.g. some authors favored splitting both *Haemoproteus* and *Leucocytozoon* into two genera (Bennett et al., 1965; Martinsen et al., 2008) and several studies found *Plasmodium* to be paraphyletic (e.g. Perkins and Schall, 2002; Outlaw and Ricklefs, 2011). All haemosporidian parasites share similar life cycles. They use blood-feeding dipterans as vectors (Garnham, 1966; Valkiūnas, 2005). Sexual reproduction occurs in the gut of the vector and the infectious sporozoites develop in the salivary glands. When the vector feeds on a vertebrate host, the sporozoites enter the blood stream and invade hepatocytes or endothelial cells. In these cells, the parasites undergo the first cycle of schizogony. Once released, the merozoites infect new cells of various tissues where they undergo another cycle of schizogony (Garnham, 1966). In contrast to most other haemosporidians, *Plasmodium* parasites also undergo schizogony in erythrocytes (Garnham, 1966; Valkiūnas, 2005). Within erythrocytes or leucocytes, the merozoites develop into gametocytes, which can then infect a new vector. Except *Leucocytozoon*, most haemosporidians form a characteristic pigment in the red blood cells called hemozoin, which is a crystalline metabolite from hemoglobin digestion by the parasite (Goldberg et al., 1990).

Before the advent of DNA sequencing methodologies, the classification of haemosporidian parasites solely relied on their morphology, their life-history traits, and the taxonomy of the infected vertebrate hosts and insect vectors (e.g., Garnham, 1966). Based on these characters, early reconstructions of haemosporidian phylogeny concluded that the most parsimonious tree comprises a monophyletic group of *Plasmodium* parasites, which exhibit the most derived traits (i.e. schizogony in the red blood cells of the vertebrate host, formation of hemozoin pigment), whereas *Leucocytozoon*, which lacks these traits, was placed at the base of Haemosporida. However, the significance of these characters for use in phylogenetic analyses had been questioned long before the first genetic sequences became available (e.g., Manwell, 1957; Garnham, 1966). Morphological traits seen under the light microscope can be distorted by preservation and only give an approximate representation of the underlying three-dimensional structure of the parasites (Martinsen et al. (2008) compared it to "systematic study of insects based on remains seen on automobile windshields"). Life-history traits, such as the production of hemozoin pigment or the types of host cells used for schizogony, could have evolved convergently on the basis of similar ecological pressures. While host switches between distantly related hosts have long been regarded as major events in the evolution of Haemosporida (Garnham, 1966), this view has been challenged by recent evidence for multiple host switches between birds and bats (Duval et al., 2007; Witsenburg et al., 2012).

A major point of contention concerning the haemosporidian phylogeny is the position of the root. Early molecular analyses were limited to single gene fragments. In a study based on the mitochondrial gene cytochrome b (*cytb*) using the piroplasmid *Theileria annulata* as outgroup, Perkins and Schall (2002) supported a basal position of *Leucocytozoon*. Hagner et al. (2007), by contrast, employed fragments of three genes (including *cytb*) for independent phylogenetic reconstructions and concluded that none of the analyzed genes alone contained sufficient phylogenetic information to resolve deep-level relationships. A multigene analysis based on four genes (Martinsen et al., 2008) resulted in a topology with high support for most splits. However, the tree was rooted with *Leucocytozoon* and did not include any non-haemosporidian outgroup taxa because the outgroup sequences were considered too divergent. To address this issue, Outlaw and Ricklefs (2011) reevaluated the dataset of Martinsen et al. (2008) using an outgroup-free molecular clock approach for rooting. In the resulting tree, Haemosporida are split into two major clades, one comprising all mammalian *Plasmodium* lineages (plus *Hepatocystis*), the other uniting the sauropsid parasites.

Originally, all avian parasites that produce hemozoin pigment but do not undergo schizogony in the red blood cells were classified as members of the genus *Haemoproteus*. Bennett et al. (1965) proposed splitting *Haemoproteus* into two genera, *Haemoproteus* and *Parahaemoproteus*. *Haemoproteus sensu* Bennett et al. (1965) comprises the parasites that use hippoboscid flies as vectors while *Parahaemoproteus* relies on mosquitoes for transmission. Molecular analyses mostly recovered these two groups of parasites as distinct lineages. However, the taxonomic status of *Haemoproteus* remained uncertain, because some studies favored a sister group relationship between both clades, thereby supporting a single genus *Haemoproteus* divided into two subgenera (Iezhova et al., 2011; Pineda-Catalan et al., 2013), while other analyses found this taxon to be paraphyletic (Martinsen et al., 2008; Witsenburg et al., 2012).

The phylogenetic placement of the bat-infecting genera *Hepatocystis*, *Polychromophilus* and *Nycteria* has proven especially troublesome. In contrast to *Plasmodium* parasites, they lack the ability to reproduce asexually in erythrocytes (blood schizogony). However, studies based on molecular data have consistently recovered them nested within *Plasmodium*. *Hepatocystis* was found to be closely associated with mammalian *Plasmodium* in numerous analyses (e.g., Escalante et al., 1998; Perkins and Schall, 2002; Martinsen et al., 2008). Witsenburg et al. (2012) expanded the four-gene dataset (Martinsen et al., 2008) to include two species of *Polychromophilus* and recovered this taxon closely related to the clade of sauropsid-infecting *Plasmodium*, similar to the results of Duval et al. (2007) and Megali et al. (2011). Schaer et al. (2013) increased the taxon sampling of bat parasites by adding various species of the genera *Plasmodium*, *Hepatocystis*, *Nycteria* and *Polychromophilus* and found *Polychromophilus* to be most closely related to a clade comprising *Nycteria* and the mammalian lineage of *Plasmodium* and *Hepatocystis*.

The majority of recent studies found *Plasmodium* to be paraphyletic with regard to the chiropteran haemosporidians (see above), Outlaw and Ricklefs (2011) even recovered the genus *Plasmodium* as a polyphyletic group and placed the mammalian *Plasmodium* lineage at the base of Haemosporida. Despite these marked differences in topology, analyses based on single genes or on variations of the four-gene dataset of Martinsen et al. (2008) have generally recovered a monophyletic group comprising all mammalian Plasmodium species (also including *Hepatocystis*). By contrast, a phylogenetic analysis of the available genome data (Pick et al., 2011) found a close relationship between the avian parasite *P. gallinaceum* and the most malignant agent of human

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