The rediscovery of malaria parasites of ungulates

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SUMMARY

Over a hundred years since their first description in 1913, the sparsely described malaria parasites (genus *Plasmodium*) of ungulates have been rediscovered using molecular typing techniques. In the span of weeks, three studies have appeared describing the genetic characterization and phylogenetic analyses of malaria parasites from African antelope (*Cephalophus* spp.) and goat (*Capra aegagrus hircus*), Asian water buffalo (*Bubalus bubalis*), and North American white-tailed deer (*Odocoileus virginianus*). Here we unify the contributions from those studies with the literature on pre-molecular characterizations of ungulate malaria parasites, which are largely based on surveys of Giemsa-reagent stained blood smears. We present a phylogenetic tree generated from all available ungulate malaria parasite sequence data, and show that parasites from African duiker antelope and goat, Asian water buffalo and New World white-tailed deer group together in a clade, which branches early in *Plasmodium* evolution. Anopheline mosquitoes appear to be the dominant, if not sole vectors for parasite transmission. We pose questions for future phylogenetic studies, and discuss topics that we hope will spur further molecular and cellular studies of ungulate malaria parasites.

Key words: malaria, ungulates, ruminants, Haemosporidia, Apicomplexa, malaria parasites.

INTRODUCTION

The first paper on ungulate malaria parasites begins off-handedly; 'It would appear from a perusal of the available literature that malaria of antelopes has not hitherto been described…', and proceeds to 'place on record' the description of a *Plasmodium* found in the blood of a captive common duiker antelope (*Sylvicapra grimmia*) in Malawi (Bruce *et al*. 1913). The word malaria was used because the antelope had an acute attack lasting 4 days, perhaps due to the stress of its captivity, and the parasite was described as *Plasmodium* based upon the morphological similarity of its intraerythrocytic stages to the human malaria parasite *Plasmodium malariae*. This same research team found another *Plasmodium* species in the common duiker (Bruce *et al*. 1915) but the organism was not revisited and named for another 50 years (Garnham, 1966; Keymer, 1966). In the ensuing years after 1913 additional ungulate *Plasmodium* parasites were described; in water buffalo (*Bubalus bubalis*) in India (Sheather, 1919), mouse deer (family Tragulidae) in Malaysia (Garnham and Edeson, 1962; Sandosham *et al*. 1962), African marshbuck (*Tragelaphus spekii*) (van den Berghe, 1937) and domestic goats (*Capra aegagrus hircus*) in Africa (de Mello and Paes, 1923). A compendium of the parasites and studies are presented in Table 1 and the global distribution of malaria parasites of ungulate hosts is shown in Fig. 1. The parasites were typically identified in the course of blood surveys of parasites of wild and splenectomized animals, as well as veterinary diagnoses of likely immunocompromised water buffalo used in the production of rinderpest immune sera (Rao, 1938; Riaz-ul-Hassan, 1953; Shastri *et al*. 1985; Kolte *et al*. 2002; Shinde *et al*. 2005). The most recent identification of a new species of *Plasmodium* in ungulates was by P.C.C. Garnham, which included the description of an infection of a single splenectomized white-tailed deer in North America from a study in 1967 (Kuttler *et al*. 1967; Garnham and Kuttler, 1980).

In the past three decades the academic conversation on ungulate malaria parasites effectively ceased, with the exception of veterinary reports on water buffalo malaria (Shastri *et al*. 1985; Kolte *et al*. 2002; Shinde *et al*. 2005); and the advent of molecular and cellular research on malaria parasites did not extend to include ungulate *Plasmodium* parasites but rather focused on the parasites of humans, non-human primates, rodents, reptiles and birds. Phylogenetic descriptions of *Plasmodium* and other Haemosporidia also ignored ungulate malaria

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Table 1. The ungulate *Plasmodium* and *Hepatocystis* species including host species, geographic region, DNA markers associated with these taxa and references

Parasite species	Host species/region	DNA markers	Comments	References
P. bubalis	Water buffalo/Asia	Whole mtDNA, *clpc*	Two species identified, types I and II	Sheather (1919), Rao (1938), Riaz-ul-Hassan (1953), Shastri *et al.* (1985), Kolte *et al.* (2002), Shinde *et al.* (2005), Templeton *et al.* (2016)
P. traguli	Mouse deer/Asia	None		Garnham and Edeson (1962), Sandosham *et al.* (1962)
P. limnotragi	Marshbuck/Africa	None	Possible *Hepatocystis*	van de Berghe (1937)
P. odocoilei	White-tailed deer/ Eastern North America	*cytb, coxI, clpc*	Molecular markers identify multiple taxa	Garnham and Kuttler (1980), Martinsen *et al.* (2016)
H. hippopotami	Hippopotamus/Africa	None	Possibly rare infections	Garnham (1958)
H. fieldi	Mouse deer/Asia	None	Possible co-infections with *Plasmodium* sp.	Garnham and Edeson (1962), Sandosham *et al.* (1962)
H. fieldi cylonensis	Mouse deer/Asia	None	Sub-species status with *H. fieldi*	Sandosham *et al.* (1962)
P. cephalophi	Duiker antelope/ Africa	Molecular markers identify multiple *Plasmodium* types in duikers, but no morphological confirmation with *P. cephalophi* or *P. brucei*		Bruce *et al.* (1913), Bruce *et al.* (1915), Keymer (1966, 1969), Boundenga *et al.* (2016)
P. brucei	Duiker antelope/ Africa	Molecular markers identify multiple *Plasmodium* types in duikers, but no morphological confirmation with *P. cephalophi* or *P. brucei*		Bruce *et al.* (1915), Keymer (1966, 1969) Boundenga *et al.* (2016)
P. caprae	Goat/Africa	Whole mtDNA and *clpc* but with no morphological confirmation. Using these markers may group with *P. bubalis* type II		de Mello and Paes (1923), Templeton *et al.* (2016)

parasites. This year, however, in the space of a few weeks, three studies have appeared describing the isolation of molecular markers for African, Asian and North American ungulates (Boundenga *et al.* 2016; Martinsen *et al.* 2016; Templeton *et al.* 2016). This review attempts to put these studies in context, reveal the importance of ungulates in the evolution of the malaria parasites, and aims to help reignite interest in a forgotten corner of malaria parasite research.

THREE INDEPENDENT STUDIES YIELD MOLECULAR DESCRIPTIONS OF UNGULATE MALARIA PARASITES

Many malaria researchers will have learned of ungulate malaria parasites while delving into Garnham's classic reference work from 1966, *Malaria Parasites and Other Haemosporidia* (Garnham, 1966). That memory, and the opportunity to study Giemsa-reagent stained slides of *Plasmodium bubalis* from a sick water buffalo from Thailand, inspired the screening of a wide inventory of ungulate blood DNA samples using a polymerase chain reaction (PCR) assay and *Plasmodium*-specific *cytb* primers, revealing the presence of malaria parasites from both water buffalo and African goats (Templeton *et al.* 2016). Independently, *Plasmodium* parasites of white-tailed deer in eastern North America were described in research led by Ellen Martinsen (Martinsen *et al.* 2016) following the serendipitous observation of a novel *Plasmodium* parasite *cytb* sequence in the course of a survey of mosquito vectors of avian malaria parasites. They then typed the vertebrate source of the mosquito blood meal by diagnostic PCR, which led them to the white-tailed deer (*Odocoileus virginianus*). Further PCR-based screening of white-tailed deer from across its range revealed the common and widespread nature of malaria parasites from white-tailed deer. The third study similarly noted a new *Plasmodium cytb* sequence, in this case through a survey of white-nosed monkey (*Cercopithecus nictitans*) bush-meat in Gabon (Ayouba *et al.* 2012). On the supposition that the monkey was an accidental host, the researchers extended the survey to include bush-meat from other species, and thus identified a diversity of malaria parasite sequences from four duiker antelope species (genus *Cephalophus*) (Boundenga *et al.* 2016). We hope that the above three surveys are just the beginning of a reawakening of molecular and cellular research on ungulate Haemosporidia.

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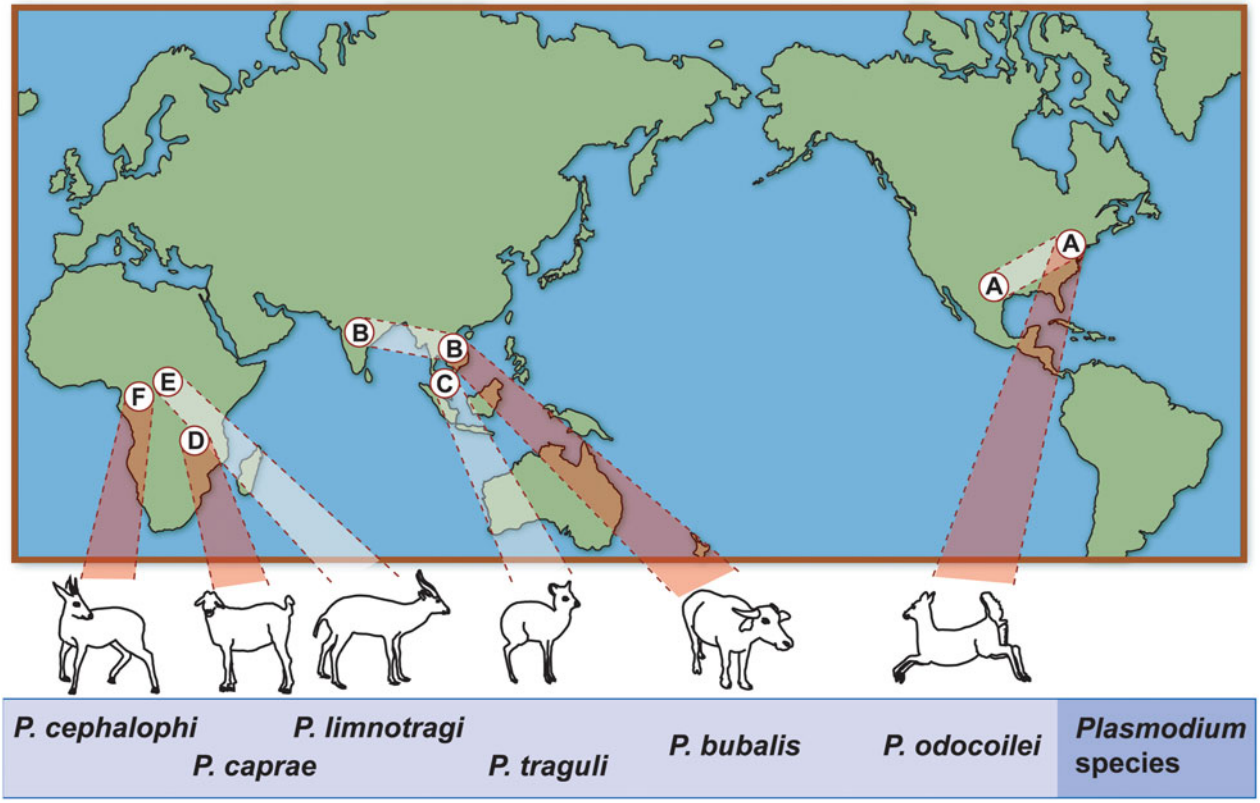

Fig. 1. Global distribution of the described ungulate malaria parasites (genus *Plasmodium*). Host animals include: (A) white-tailed deer, (B) water buffalo, (C) mouse deer, (D) domestic goat, (E) African marshbuck and (F) duiker antelope (*Sylvicapra* and *Cephalophus* spp.). Parasite species are listed below their respective hosts. Red shading indicates molecular typing of the malaria parasites (Boundenga *et al*. 2016; Martinsen *et al*. 2016; Templeton *et al*. 2016).

MOLECULAR-BASED INSIGHTS INTO UNGULATE *PLASMODIUM* EVOLUTION AND DIVERSITY

The three studies utilized molecular markers to generate phylogenetic trees, which uniformly placed ungulate malaria parasites as a clade closely related to but distinct from other mammalian *Plasmodium* (Boundenga *et al*. 2016; Martinsen *et al*. 2016; Templeton *et al*. 2016). Here we combine these newly available ungulate malaria parasite sequences to construct a new tree (Fig. 2), which includes sequence data from the mitochondrial cytochrome b (*cytb*) and cytochrome oxidase I (*coxI*) genes and the plastid caseinolytic protease gene (*clpc*) (Boundenga *et al*. 2016; Martinsen *et al*. 2016; Templeton *et al*. 2016). Phylogenies and nodal support values were estimated by Bayesian and Maximum Likelihood methods per Martinsen *et al*. (2016). Malaria parasites infecting Asian water buffalo, African duiker antelope and domestic goat and North American white-tailed deer group together within a single clade, with the duiker and water buffalo parasites interspersed amongst each other indicative of multiple colonization events of these host species. The diversity of *Plasmodium* parasites revealed from these three studies is greater than the number of morphological species traditionally described by morphology from these ungulate host species. For example, we now know by molecular analysis of sequence data that two *Plasmodium* species infect water buffalo (Fig. 2B), but only one parasite, *P. bubalis*, is described in the literature. Ungulate malaria parasite diversity is likely to increase with further surveys, making it difficult if not impossible to assign molecular sequences with historical species names. The situation will become further muddied if the parasites are found to be promiscuous with respect to ungulate hosts.

MOSQUITOES ARE THE LIKELY VECTORS OF UNGULATE MALARIA PARASITES

Ungulate malaria parasites thus far group within a single clade, suggesting that fundamental aspects of their natural histories might be conserved, such as their use of a specific arthropod transmission vector. Studies on anopheline mosquitoes feeding on water buffalo and mouse deer (Toumanoff, 1939; Wharton *et al*. 1963) proposed that they are the definitive host of ungulate *Plasmodium*. *Mansonia crassipes* was reported as a vector for the mouse deer malaria parasite, *Plasmodium traguli* (Warren *et al*. 1964), in addition to *Anopheles umbrosus* (Wharton *et al*. 1963). Two of the recent molecular studies included mosquito surveys, and both implicated *Anopheles* spp. in the transmission of

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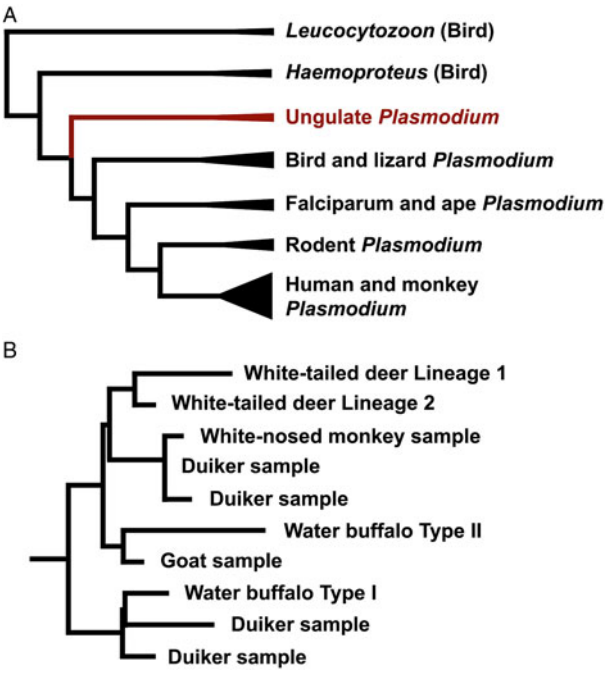

Fig. 2. Unification of ungulate malaria parasites within a single clade. (A) We inferred a new tree for Haemosporidia using the mitochondrial cytochrome *b* (*cytb*) and cytochrome oxidase I (*coxI*) genes and the plastid caseinolytic protease (*clpc*) gene sequences; plus *cytb* sequences from duiker samples. Phylogenies and nodal support values were estimated by Bayesian and Maximum Likelihood methods as in Martinsen *et al*. (2016). The ungulate malaria parasites group within a single clade, shown in red, which branches early in haemosporidian evolution. The clade is expanded in (B) to show the relationships between the ungulate malaria parasites. The Bayesian posterior probability and Maximum Likelihood bootstrap support values for this clade were 1.00 and 100% respectively. The grouping of the white-nosed monkey sample within this clade is discussed further in references Boundenga *et al*. (2016) and Templeton *et al*. (2016).

ungulate malaria parasites; *Anopheles punctipennis* in the case of the white-tailed deer parasites, and various *Anopheles* spp. collected from Gabonese forests regarding parasites infecting duiker antelope species (Boundenga *et al*. 2016; Martinsen *et al*. 2016). Further surveys of wild caught mosquitoes, and establishment of experimental feeding assays on infected animals using a variety of mosquitoes and other biting insects, will confirm these observations. Below, these points are reiterated with respect to the ungulate *Hepatocystis* parasites.

PRE-ERYTHROCYTIC AND ERYTHROCYTIC STAGES OF UNGULATE MALARIA PARASITES

Because ungulate malaria parasites branched prior to the clade containing avian, reptile and the other mammalian *Plasmodium*, it is of interest to understand the host tissues that support pre-erythrocytic development. For example, the sporozoites of avian malaria parasites develop within the cells of various tissues prior to the establishment of the erythrocytic cycle (Valkiūnas, 2005), in contrast to the pre-erythrocytic development within hepatocytes of all characterized mammalian malaria parasites, including mouse deer *Plasmodium* (Garnham and Edeson, 1962; Sandosham *et al*. 1962). Knowledge is lacking on the complete life cycle of ungulate malaria parasites within their vertebrate hosts. It also has not been determined if ungulate malaria parasites have hypnozoite stages. This is relevant to the *Plasmodium* of white-tailed deer, as mosquitoes are absent for much of the year, and the parasite must either persist in its vertebrate host through a chronic blood stage infection or recrudesce via activation of latent hypnozoites.

The parasites of ungulates appear to share the life history trait of extremely low or sub-microscopic blood parasitaemias, persisting as chronic, occult infections in the blood of their ungulate hosts. Schizonts are frequently so scarce that distinguishing *Hepatocystis vs Plasmodium* blood stage infections is difficult (Keymer, 1966). As with the original species description of *P. odocoilei* from a single white-tailed deer, Martinsen *et al*. (2016) documented extremely low blood parasitaemia and oftentimes undetectable levels of the malaria parasites of this host species by visualization of blood smears from positive animals sampled during warm months. This likely explains why *P. odocoilei* defied all attempts at rediscovery for decades despite a multitude of white-tailed deer blood parasite studies by light microscopy methods. The occult or subpatent status of the parasite might be punctuated by elevated parasite blood burdens and gametocytaemia for the facilitation of transmission during the mosquito season. Alternatively, the parasite may maintain infectivity at sub-microscopic levels of parasitaemia, as has been shown for malaria parasites in humans (reviewed in Okell *et al*. 2009) and for many other mammalian malaria parasites including the two species of common duiker *Plasmodium* (Garnham, 1966). The duiker parasites, *Plasmodium cephalophi* and *Plasmodium brucei*, likewise resisted efforts at their rediscovery for over 50 years due to their light parasitaemia, until researchers splenectomized duikers to recover the parasites (Keymer, 1966). *Plasmodium bubalis* also appears to be occult; for example, in a PCR *cytb* survey of 144 blood samples from water buffalo in the Mukdahan Province of Thailand, 45% were positive for *Plasmodium*, but only one of these samples yielded microscopic detection of the parasite in Giemsa reagent-stained blood smears (Templeton *et al*. 2016). The occult nature of the blood stages of ungulate malaria parasites emphasizes the need for molecular-based methods in the discovery and rediscovery of the malaria parasites of ungulates.

Descriptions of Giemsa reagent-stained blood smears of ungulate malaria parasites indicate that

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great diversity exists within the clade with respect to morphologies of the intraerythrocytic stages. For example, *P. bubalis* parasites possess long, bar-shaped hemozoin crystals (Sheather, 1919; Templeton *et al*. 2016), also observed in *P. cephalophi* (Keymer, 1966), whereas the granules are small in *P. odocoilei* (Garnham and Kuttler, 1980; Martinsen *et al*. 2016). The latter parasite shares with *P. cephalophi* profound enlargement of the host erythrocyte, and spiked processes on the host erythrocyte, which Garnham noted are reminiscent of similar structures which provide the Latin name for the bat haemosporidian, *Nycteria medusiformis* (Garnham and Kuttler, 1980). *Plasmodium odocoilei* also imparts a pink discoloration and the accumulation of large vacuoles within the erythrocyte (Garnham and Kuttler, 1980).

THE PARAPHYLETIC NATURE OF HAEMOSPORIDIA AND QUESTIONS REGARDING UNGULATE *PLASMODIUM*

The *Plasmodium* clade within Haemosporidia is paraphyletic, in that it includes the bat malaria parasites *Nycteria* and *Hepatocystis* (Escalante *et al*. 1998; Perkins and Schall, 2002; Martinsen *et al*. 2008; Witsenburg *et al*. 2012; Schaer *et al*. 2013; Borner *et al*. 2016; Templeton *et al*. 2016). In contrast to *Plasmodium* these parasites do not possess the capacity for intraerythrocytic schizogony, and are vectored by biting flies rather than mosquitoes. The three recent studies of ungulate malaria parasites present phylogenetic trees, which differ in the placement of ungulate malaria parasites within Haemosporidia, and their relationship with *Polychromophilus*, another haemosporidian parasite of bats (Fig. 3). Thus it formally remains a question if ungulates represent the basal branch of Haemosporidia in mammals, with the avian parasites such as *Haemoproteus, Parahaemoproteus* and *Leucocytozoon* representing the root of the Haemosporidia. All three studies support a close relationship of ungulate malaria parasites to *Polychromophilus*, which lacks erythrocyte schizogony, has merogony in Kupffer and endothelial cells of multiple tissues, and is vectored by bat flies of the family Nycteribiidae. More robust sampling of DNA markers from *Polychromophilus* spp., as well as other haemosporidian groups including the ungulate malaria parasites, will aid in obtaining higher resolution trees to resolve the base of the Haemosporidia.

UNGULATE *HEPATOCYSTIS* PARASITES REMAIN UNCHARACTERIZED

An ungulate *Hepatocystis* parasite, *Hepatocystis hippopotami*, has been described in the hippopotamus (Garnham, 1958) and two species, *Hepatocystis fieldi* and *Hepatocystis fieldi ceylonensis*, in the mouse deer (Garnham and Edeson, 1962; Sandosham *et al*. 1962; Dissanaike, 1963). They differ from ungulate *Plasmodium* in the observation of merocysts on the surface of the liver and the absence of intraerythrocytic schizogony. Mouse deer have been observed carrying both *Hepatocystis* and *Plasmodium* (*P. traguli*) infections, which can be differentiated based upon gametocyte morphology (Dissanaike, 1963). Thus the puzzle of ungulate Haemosporidia parasites has an unsolved component – the molecular phylogenetic placement of ungulate *Hepatocystis*. The Asian mouse deer might be the best source of parasite material with which to pursue research on this topic, as infections were reported to be more common than those of the hippopotamus. It is of interest to determine if ungulate *Hepatocystis* and *Plasmodium* diverged from a common ungulate parasite ancestor, or if they are the descendants of independent host-switches to ungulates. Related to this, the insect vector of ungulate *Hepatocystis* also has not been described; its identity, coupled with the phylogenetic placement of the *Hepatocystis*, might indicate if there was also host switch in the vector.

THE VALUE OF UNGULATE *PLASMODIUM* FOR GENOME STUDIES

Whole genome sequence information is invaluable in the study of the evolution of malaria parasites. For example, understanding the origin and diversification of parasite-encoded proteins, which mediate host interactions, such as the *Plasmodium*-encoded erythrocyte surface receptors PfEMP1, PIR and SURFIN, would be greatly informed by the genome sequences of avian and ungulate *Plasmodium*. The recent completion of genome sequence for *Haemoproteus tartakovskyi* (Bensch *et al*. 2016) will be valuable in describing the evolution of Haemosporidia virulence genes. Similarly, ungulate malaria parasites are of interest for genome projects because of their distant relationship to other mammalian parasites. The low parasitaemias common to these parasites is challenging in terms of preparing quantities of DNA sufficient for whole genome sequencing, but might be overcome by infection of splenectomized animals, through *in vitro* culture, or via selective whole genome amplification (Sundararaman *et al*. 2016). The goat malaria parasite, *P. caprae*, might be useful because of the availability of goats, which are easy to splenectomize, and to restrain for bloodletting. Bovinized splenectomized SCID mice, in which the mouse blood component is replaced by frequent intraperitoneal injections of bovine blood, might also allow sufficient production of ungulate *Plasmodium* for genome studies. This system has been successfully used to propagate *Babesia* parasites (Arai *et al*. 1998). Tissue culture adaptation might also be attempted with either *P. caprae* or *P. bubalis*, since blood draws of large volumes are possible from

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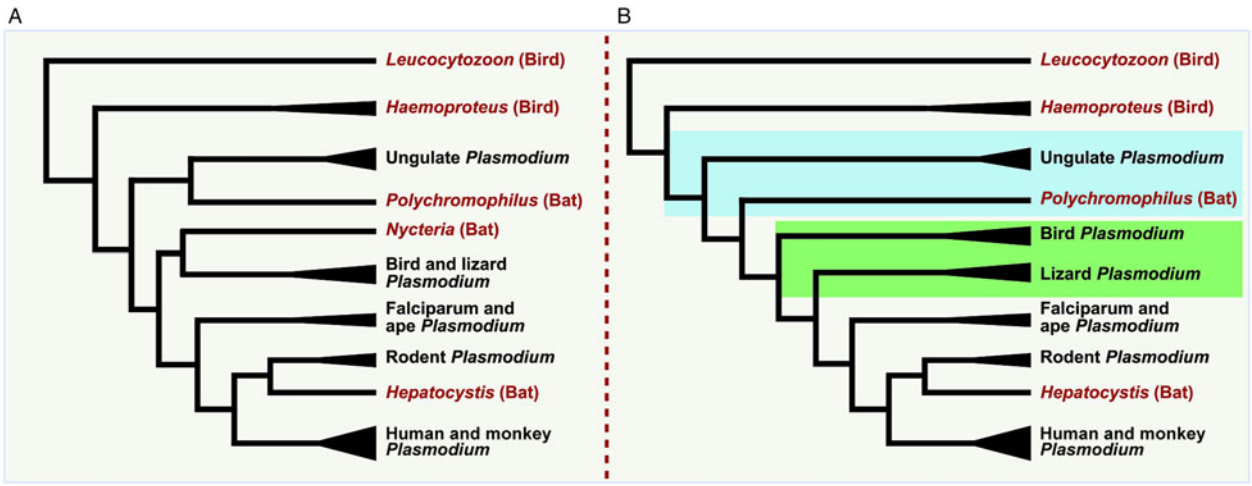

Fig. 3. The paraphyletic nature of Haemosporidia and the relationship of ungulate malaria parasites to *Polychromophilus*. *Plasmodium* species are in black font, and other genera in red font. (A) Topology from Martinsen *et al*. (2016). (B) Topology from Templeton *et al*. (2016), in which the relationship of ungulate *Plasmodium* and *Polychromophilus* was not well-resolved (indicated by a blue box). In other trees inferred from whole mtDNA sequences, which did not include *Polychromophilus* (Templeton *et al*. 2016) the bird and lizard *Plasmodium* species grouped within a single well-supported clade (indicated by a green box).

both host animals. Recent advances in whole genome sequencing have greatly lowered the cost, and requirements for abundance and purity of starting material; and it is anticipated that these methods will benefit the sequencing of the above challenging parasites. Whole genome amplification methods (for example, see Oyola *et al*. 2014) might also allow whole or high coverage genomes for annotation of virulence genes and sequences for phylogenetic analyses.

VETERINARY AND WILDLIFE MANAGEMENT ASPECTS OF UNGULATE MALARIA PARASITES

The world population of water buffalo was approximately 200 million head in 2014, with over 95% located in Asia, according to databases at the Food and Agriculture Organization (FAO) of the United Nations (http://faostat3.fao.org). Water buffalo can be subdivided into 2 subspecies: river buffalo, constituting approximately 70% of the population, and swamp buffalo. The population of goats was over one billion head in 2014 and distributed worldwide. Together water buffalo and goat contribute a significant share of global milk; and in several countries are the major milk and meat producing animals. Successful infrastructure development projects across Asia have resulted in considerable shipping of water buffalo both within and across borders. The livestock frequently spend long periods of time in transport and quarantine, and are intermixed with local stock. Consequently, any pathogens that compromise food production, whether they be of bacterial, viral or protozoan nature, are threats to both local economies and international food security (Godfray *et al*. 2010).

Templeton *et al*. (2016) reported that *Plasmodium* is widespread in water buffalo of the Mukdahan Province of Thailand, with up to 48% prevalence using a sensitive *Plasmodium*-specific PCR assay. They also observed one case of mortality of a malarial buffalo, although it was not determined if the malaria parasite was an opportunistic infection and not the causative agent of mortality. Malaria has been observed in likely immunocompromised water buffalo used in the production of rinderpest and anthrax immune sera, including deaths possibly attributed to the parasite (Rao, 1938; Riaz-ul-Hassan, 1953; Shastri *et al*. 1985; Kolte *et al*. 2002; Shinde *et al*. 2005). Although we feel that it is unnecessary at this time to raise public concern regarding water buffalo malaria parasites, it is important to develop rapid diagnostic tests (RDT) for research purposes, and to formulate epidemiological questions to be addressed by field surveys using RDT and PCR. Diagnostic assays might be employed in veterinary cases that have unknown causes or suspected involvement of malaria parasites. Templeton *et al*. (2016) reported screening of 53 samples of goat blood for *Plasmodium*, from Zambia, and identified a single positive sample. In a screen of 46 goat samples from Vietnam positive samples were not identified. These assays did not contain positive controls for DNA quality, since we were looking for positive samples rather than an epidemiological survey. To our knowledge malaria parasites have not been described in cows (*Bos taurus*). In a screen of 140 samples from Vietnam we did not identify *Plasmodium*-positive samples. Thus it is necessary to extend such surveys to include more goats and cows, globally, in order to determine the distribution and prevalence of malaria parasites in these domestic ruminants. It is also of

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interest to develop and include malaria diagnostics as a veterinary consideration for undiagnosed ailments in goats, and in cows if surveys identify *Plasmodium* parasites. In summary, the global health and economic burden of malaria parasite infections in ungulate hosts of domestic, such as water buffalo and goat, and wild, such as white-tailed deer and duiker antelope species, are unknown but future study will be aided by the further development of molecular markers and the formulation of topics and questions for study.

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