What Fossils Reveal

About the Protozoa Progenitors, Geographic Provinces, and Early Hosts of Malarial Organisms

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In 1991, Robert S. Desowitz asked, "Did the primitive malaria begin as a parasite of some prehistoric reptile that later was picked up by a mosquito, or was it first a parasite of the mosquito that later became established in the reptile?" This question has been debated for years and is addressed in the present work in light of the fossil record of malarial organisms (Haemosporidia). The general consensus is that malaria evolved as a parasite of vertebrates (Manwell 1961, Desportes and Schrével 2013, Mattingly 1983); however, Huff (1945) felt that the malaria progenitor originated with the vector, and in their discussion of malaria evolution, Di Fiore et al. (2009) recognized that in the digenetic malarial life cycle, the vector is the definitive host and the vertebrate the intermediate host. This question has also been addressed with molecular data, but thus far, only very small DNA segments have been analyzed, resulting in incongruous and poorly resolved gene trees (Di Fiore et al. 2009). In the present analysis, fossils are used to determine the progenitors, ancient hosts, and original geographic provinces of malarial organisms.

Malaria, 100 Million Years Ago

Malarial vectors are ancient, as demonstrated by the early mid-Cretaceous female biting midge (Diptera: Ceratopogonidae) of the extinct genus *Protoculicoides* infected with *Paleohaemoproteus burmacis* Poinar and Telford (2005) (Fig. 1). The abdomen of the *Protoculicoides* vector had cleared enough to reveal 35 oocysts of *P. burmacis* in its body cavity (Fig. 2). The oocysts contained developing sporozoites, some of which had broken out of their cysts (Fig. 3), thus showing that the pattern of sexual reproduction (sporogony) was already established in vectors at that time.

Determination of the vertebrate host of *P. burmacis* was based on characters of the *Protoculicoides* vector. Antennal features indicated that the host was cold-blooded. That the earliest vertebrates infected with malaria were reptiles is supported by the cytochrome b-based phylogenetic tree of Yotoko and Elisei (2006), showing that the most likely ancestral hosts of *Plasmodium*, *Haemoproteus*, and *Hepatocystis* malaria were Squamata.

Based on the above association and the fossil record of biting midges, the

Fig. 1. One hundred million-year-old *Protoculicoides* biting midge containing numerous oocysts (arrow) of the malarial parasite *Paleohaemoproteus burmacis* in Myanmar amber.

Ceratopogonidae were probably the earliest malarial vectors, with reptiles as the vertebrate hosts (Telford 2009). Other insect vectors of reptilian malaria are sand
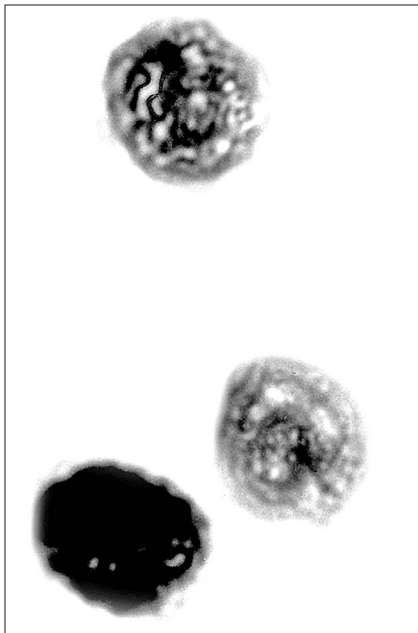
Fig. 2. Detail of oocysts of *Paleohaemoproteus burmacis* in various stages of development in the abdomen of the *Protoculicoides* biting midge shown in Fig. 1.
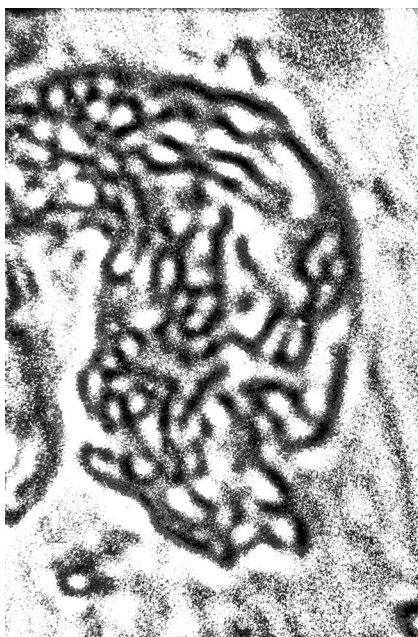

Fig. 3. Detail of sporozoites of *Paleohaemoproteus burmacis* in the abdomen of the *Protoculicoides* biting midge shown in Fig. 1.

flies (Phlebotomidae), mosquitoes (Culicidae), and tabanids (Tabanidae) (Telford 2009). Of these, biting midges are the most ancient, with fossils dating back to 140 million years ago (Kaddumi 2007).

Did Malaria Progenitors Originate in Insects or Vertebrates?

Sexual reproduction (sporogony) is a basic process in any organism's life cycle, and it only occurs in the vector in malarial organisms. Asexual malarial cycles (schizogony and merogony) that replicate genetic material from a mother cell only occur in the vertebrate host. The two most likely groups of protozoa that could function as malarial progenitors are the Coccidia and the Gregarinida. Together with the Haemosporidia, these three groups have been placed in the phylum Apicomplexa, which is an ARL-V lineage identified from plastid environmental surveys (Keeling and Rayner 2015). All three groups are characterized by a sporozoite infective stage, a sexual cycle (sporogony), and asexual cycles (schizogony and merogony) (Manwell 1961, Desportes and Schrével 2013, Mattingly 1983).

If the malarial progenitor co-evolved with vertebrates, then it was probably a coccidial parasite, since the vertebrate host range of coccids is quite extensive (Manwell 1961). In contrast, only a few Coccidia parasitize insects and most infections are considered accidental (Brooks 1988, Steinhaus 1949). Only a single dipteran is known to be infected by a coccidian, namely a larval crane fly, *Ithania wenrichi* Ludwig (Brooks 1988, Steinhaus 1949, Poinar and Thomas 1984). Fleas and mites can transmit some coccidian parasites of vertebrates, but they are mechanical vectors and the protozoa do not develop within their bodies (Steinhaus 1949, Mullen and Durden 2002). There are no coccidian parasites of mosquitoes, biting midges, or other dipteran vectors of malaria (Steinhaus 1949, Poinar and Thomas 1984).

If the malarial progenitor co-evolved with the vector, then it could have been a gregarine parasite. Gregarines are considered the most primitive group of the phylum Apicomplexa and include the orders Archigregarinorida, Neogregarinorida, and Eugregarinorida (Desportes and Schrével 2013). Gregarines do not infect vertebrates, but parasitize a wide range of marine and terrestrial invertebrates, including all of the major groups of malarial vectors (Desportes and Schrével 2013). The Neogregarinorida contain two families that parasitize Diptera. *Schizocystis gregarinoides* Léger (of the family Schizocystidae) is a parasite of biting midges. Species of the genus *Caulleryella*, in the Caulleryellidae, parasitize mosquitoes. The neogregarine life cycles include an asexual cycle involving the production of merozoites (Desportes and Schrével 2013), resembling that of the malaria cycle.

In the Eugregarinorida, representatives of the genera *Stylocystis*, *Taeniocystis*, and *Allantocystis* in the family Actinocephalidae parasitize Ceratopogonidae, and members of the genus *Ascogregarina* of the family Ascogregarinidae parasitize mosquitoes (Desportes and Schrével 2013). Representatives of both eugregarines and neogregarines that infect culicids and biting midges could have co-evolved as parasites of early lineages of these dipterans.

An example of a eugregarine parasite that parasitizes mosquitoes today and has a life cycle with stages roughly equivalent to those of malarial organisms is *Ascogregarina culicis* (Sanders and Poinar 1973). However, instead of having sexual and asexual life cycles in different

Fig. 4. Trophozoites of the mosquito gregarine parasite *Ascogregarina culicis* developing in the gut wall of a larva of *Aedes sierrensis*.
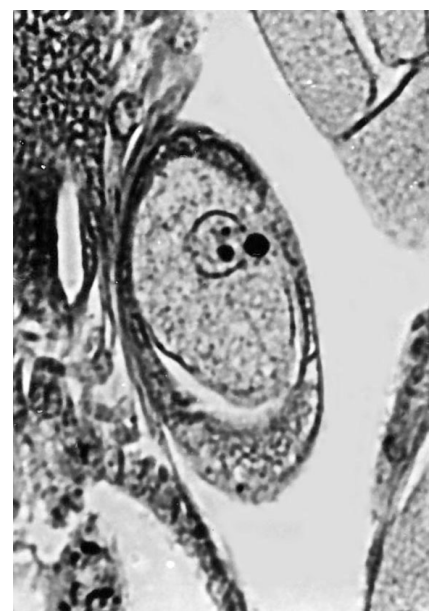

Fig. 5. Gamont of the mosquito gregarine parasite *Ascogregarina culicis* developing in the Malpighian tubules of a pupa of *Aedes sierrensis*.
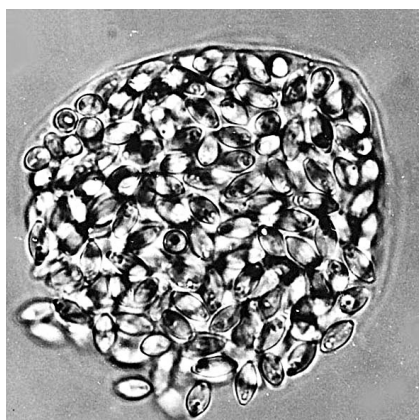
Fig. 6. Mature zygote of the mosquito gregarine parasite *Ascogregarina culicis* liberating spores containing sporozoites in the lumen of the Malpighian tubules of an adult *Aedes sierrensis*.
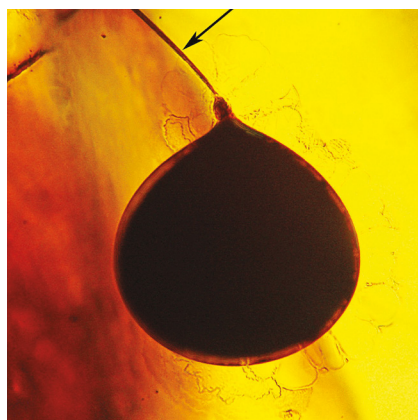

Fig. 7. Gametocyst of the gregarine *Primigregarina burmanica* in early mid-Cretaceous Myanmar amber. Arrow shows extruded spore duct filled with spores containing sporozoites.

hosts, the different stages of *Ascogregarina* often occur in larvae, pupae, and adult mosquitoes, respectively. Sporozoites of *Ascogregarina* invade and form trophozoites in the midgut cells of the mosquito larva (Fig. 4). In the pupal stage, gamonts invade the Malpighian tubules and form gametocysts that produce micro- and macro-gametes (Fig. 5). In the adult mosquito, sexual union of the gametes occurs, with zygote development and the release of sporozoites into the environment via the hindgut and anus (Fig. 6). The sporozoites are ingested by a larva to continue the cycle (Mullen and Durden 2002). Essentially, *Ascogregarina* has its sexual stages in the adult mosquito and asexual stages in the mosquito's larval and pupal stages. *Plasmodium* species also have the sexual cycle in the adult mosquito but the asexual cycles in a vertebrate. If malarial progenitors were gregarines that infected vectors, either there were no original asexual cycles (as in the eugregarines) or asexual cycles were present (as in the neogregarines), but were later lost and acquired by vertebrate hosts.

Desportes and Schrével (2013) mention some features that gregarines and *Plasmodium* have in common. Trophozoites of the Archigregarina species *Selenium hollande* are structurally similar to the micropyle in *Plasmodium*. Estimates of the intramembraneous particles (IMPS) for the trophozoite membranes of *Gregarina blaberae* are similar to estimates of IMPS in merozoites of *Plasmodium knowlesi*. Class XII proteins of the myosin family in gregarines also occur in *Plasmodium*. Kinetochores in the gregarines *Lecudina tuzetae* and *Grebnickiella gracilis*

are similar to those in *Plasmodium berghei*. The biogenesis of flagella in male gametes of *L. tuzetae* is similar to that in *Plasmodium*. Synaptonemal complexes in sporoblasts of *Grebnickiella gracilis* also occur in zygotes of *Plamsodium berghei*. During sporozoite differentiation of Eugregarines, a spatio-temporal process similar to the one demonstrated in the biogenesis of *Plasmodium* sporozoites appears to occur.

Unfortunately, there are no molecular studies comparing the genomes of malarial organisms with those of coccids and gregarines (Desportes and Schrével 2013, Di Fiore et al. 2009). This will be a challenge because the technique of using molecular markers to evaluate the relationships among the various types of malaria is still far from complete (Perkins
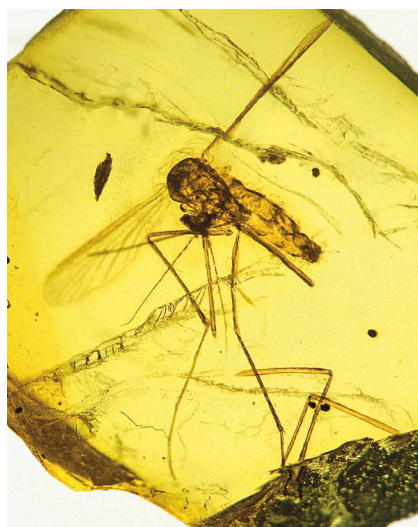

Fig. 8. The Tertiary mosquito *Culex malariager* infected with the malarial parasite *Plasmodium dominicana* in Dominican amber.

2014). Gregarines are an ancient group and probably much older than their fossil record of some 100 million years indicates (Poinar 2012; Fig. 7).

Ancient *Plasmodium* Malaria

Malarias of the genus *Plasmodium* that infect birds and mammals are transmitted by anopheline mosquitoes (Culicidae). The oocysts occur in the gut wall, rather than the body cavity, and emerging sporozoites migrate to the salivary ducts, where they are transmitted back to a vertebrate host (Garnham 1966).

This type of malaria was discovered in the mid-Tertiary culicine mosquito, *Culex malariager* Poinar, in Dominican amber (Poinar 2005A, 2005B; Fig. 8). Inside the vector's body were gametes, an ookinete and oocysts of *Plasmodium dominicana* Poinar (2005B) in various stages of development (Fig. 9), including some mature cysts liberating sporozoites (Fig. 10). Aside from being the first fossil record of *Plasmodium* malaria, the fossil demonstrated that this genus was established in the New World at least 15 million years ago, thus putting the history of malaria in the New World in a different perspective.

The large, pedunculated cysts of *P. dominicana* align it with the extant *Plasmodium juxtanucleare* that infects birds (Poinar 2005B). Since it is possible for either culicine or anopheline mosquitoes to act as bridge vectors, they could have transmitted *P. dominicana* or other *Plasmodium* species to simians (Garnham 1966), thus making it possible that ancestral lineages of these New World
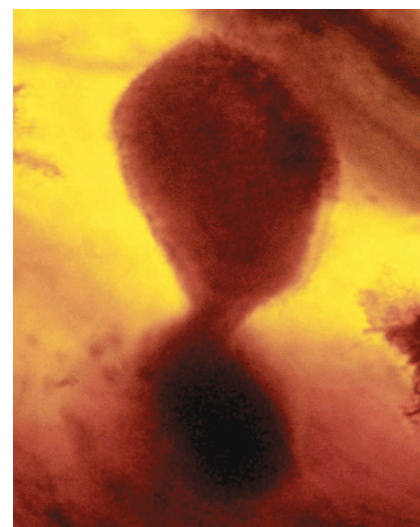

Fig. 9. Developing oocyst of the Tertiary *Plasmodium dominicana* in the abdomen of *Culex malariager* in Dominican amber.
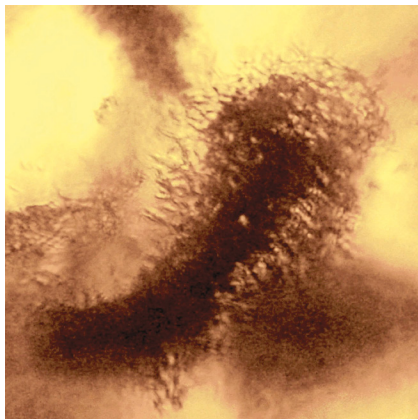
Fig. 10. Sporozoites emerging from a mature oocyst of the Tertiary *Plasmodium dominicana* in the body cavity of *Culex malariager* in Dominican amber.

Fig. 11. *Anopheles dominicanus* in Tertiary Dominican amber.

Plasmodium species could have evolved into *P. brasilianum*, which infects 12 genera of New World monkeys (di Fiore et al. 2009). This species of simian malaria does not occur anywhere else in the world (Garnham 1966) and is closely related to (and likely evolved into) the human parasite *Plasmodium malariae*, which infects both simians and humans (di Fiore et al. 2009).

The presence of *P. dominicana* lends support to the premise that *Plasmodium* malaria was carried from birds to mammals (including simians and hominoids) and eventually to humans when they entered South America. The presence of anophelines in the mid-Tertiary is demonstrated with the Dominican amber *Anopheles dominicanus* Zavortink & Poinar (Zavortink and Poinar 2000; Fig. 11). It and subsequent lineages could have served as vectors of New World simian and hominoid malaria. The age, origin, and subsequent dispersal of *Plasmodium* malaria in both the New and Old Worlds is controversial, primarily due to the rarity of fossil evidence. Various ages proposed for the origin of human malaria range from 15,000 to 8 million years (Pennisi 2001).

Conclusions

Based on the host range and life cycle of representatives of the Coccidia and Gregarinida, the progenitor for malarial organisms (Haemosporidia) is considered to have evolved from gregarine protozoa. The gregarine parasite could have already infected lineages of the vectors before they became blood-feeders, with the two organisms undergoing a long period of co-evolution. Malarial organisms probably originated as monogenetic parasites with a merozoic cycle (schizogony) as well as the sporogonic cycle (gamogony) in the insect host, similar to the cycles found in extant Archiogregarina and Neogregarinida (Desportes and Schrével 2013). At some later date, sporozoites were introduced into vertebrates during blood feeding, and some survived long enough to establish asexual cycles in vertebrate tissues.

Fossil evidence and molecular studies indicate that the original vertebrate hosts of malarial or plasmodid organisms were reptiles; thus, the mid-Cretaceous biting midge infected with *P. burmitis* could well represent an early prototype of a malarial vector–vertebrate association.

Acknowlegments

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