3-7-1995

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Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5793–5797, June 1995
Evolution

Evolutionary origin of *Plasmodium* and other Apicomplexa based on rRNA genes

(molecular evolution/protist evolution/origin of malaria/coccidia/dinoflagellates)

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Contributed by Francisco J. Ayala, March 7, 1995

ABSTRACT **We have explored the evolutionary history of the Apicomplexa and two related protistan phyla, Dinozoa and Ciliophora, by comparing the nucleotide sequences of small subunit ribosomal RNA genes. We conclude that the *Plasmodium* lineage, to which the malarial parasites belong, diverged from other apicomplexan lineages (piroplasmids and coccidians) several hundred million years ago, perhaps even before the Cambrian. The *Plasmodium* radiation, which gave rise to several species parasitic to humans, occurred ≈129 million years ago; *Plasmodium* parasitism of humans has independently arisen several times. The origin of apicomplexans (*Plasmodium*), dinoflagellates, and ciliates may be >1 billion years old, perhaps older than the three multicellular kingdoms of animals, plants, and fungi. Digenetic parasitism independently evolved several times in the Apicomplexa.**

The Apicomplexa is a large and complex protist phylum, consisting of nearly 5000 described species, with as many as 60,000 yet to be named (ref. 1, pp. 1–6; see also refs. 2 and 3). The apicomplexans are all parasites, characterized by the "apical complex," a structure that inspired the phylum's name. Many species have economical and medical importance, among them the malarial genus *Plasmodium*, one of the greatest causes of mortality in the world.

The taxonomy and phylogeny of the Apicomplexa have been the subject of controversy and frequent revision. At stake are such matters as the evolutionary origin of *Plasmodium*—whether it derives from gut parasites of vertebrates or from originally monogenetic (monoxenous) parasites of dipterans (4–8).

We have analyzed the small subunit (SSU) rRNA genes from 20 species that belong to three classes (we follow the taxonomy of ref. 2): Hematozoea (where *Plasmodium* is included), Coccidea, and Perkinsidea. We use as outgroups 10 species from two related phyla—Dinozoa (dinoflagellates) and Ciliophora (ciliates).

We conclude that the *Plasmodium* lineage diverged from other Apicomplexa several hundred million years ago, perhaps earlier than the Cambrian, before the vertebrates (chordates) originated from their ancestral invertebrate lineage. The Apicomplexa phylum is very ancient, perhaps as old as the three multicellular kingdoms plants, fungi, and animals.

MATERIALS AND METHODS

The 20 Apicomplexa species (Table 1) represent three of the four classes that make up the phylum (2). The Apicomplexa phylum of Corliss (2) is essentially identical to the array classified earlier as Sporozoa (19), except for the inclusion of *Perkinsus*, which remains controversial. We include 14 species of Hematozoea (5 species of *Plasmodium*, order Haemosporida, and 9 species of the order Piroplasmida). The piroplasms are often considered the sister clade of the *Plasmodium* lineage (20). We also include 5 species of the class Coccidea, from which the *Plasmodium* lineage may have originated (1, 20, 21), and 1 species of the class Perkinsidea. The 10 species used as outgroups (Table 2) belong to the phyla Dinozoa and Ciliophora, akin to the Apicomplexa to the extent that Cavalier-Smith (29) has included all three in the parvkingdom Alveolata.

We have aligned the 30 SSU rRNA sequences by means of the CLUSTAL-V program (30), with manual editing for maximizing similarity between the sequences. The final alignment consists of 1550 sites (of ≈1800 in each sequence).

Table 1. Twenty Apicomplexa species and their hosts

Code	Species	Intermediate host	Definitive host or vector
	Class Hematozoa, order Haemosporida		
Pfa	*Plasmodium falciparum*	Human	Mosquito
Pma	*Plasmodium malariae*	Human	Mosquito
Pvi	*Plasmodium vivax*	Human	Mosquito
Pbe	*Plasmodium berghei*	Rodent	Mosquito
Pga	*Plasmodium gallinaceum*	Bird	Mosquito
	Class Hematozoa, order Piroplasmida		
Bbo	*Babesia bovis*	Cattle, deer	Tick
Bca	*Babesia canis*	Canid	Tick
Bcb	*Babesia caballi*	Equid	Tick
Beq	*Babesia equi*	Equid	Tick
Cfe	*Cytauxzoon felis*	Felid	Unknown
Tbu	*Theileria buffeli*	Buffalo	Tick
Tpa	*Theileria parva*	Cattle	Tick
Tta	*Theileria taurotragi*	Cattle	Tick
Tun	*Theileria* sp.	Antelope	Tick?
	Class Coccidea		
Sgi	*Sarcocystis gigantea*	Sheep	Canid
Nca	*Neospora caninum*	Unknown	Canid
Tgo	*Toxoplasma gondii*	Mammal	Felid
Cpa	*Cryptosporidium parvum*	None	Rodent
Cwr	*Cryptosporidium wrairi*	None	Rodent
	Class Perkinsidea		
Per	*Perkinsus* sp.	None	Oyster

Accession nos. and references are as follows: Pfa, M19172 (9); Pma, M54897 (10); Pvi, X13926 (11); Pbe, M14599 (12); Pga, M61723 (13); Bbo, L19078 (14); Bca, L19079 (14); Bcb, Z15104 (14); Beq, Z15105 (14); Cfe, L19080 (14); Tbu, Z15106 (14, 15); Tpa L02366 (14, 15); Tta, L19082 (14, 15); Tun, L19081 (14, 15); Sgi, L24384; Nca, L24380 (16); Tgo, M97703 (17); Cpa, L16996; Cwr, U11440; Per, L07375 (18).

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Abbreviations: ML, maximum likelihood; My, million years; NJ, neighbor–joining; SSU rRNA, small subunit rRNA.

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Table 2. Ten species of dinoflagellates and ciliates, with accession nos. for SSU rRNA sequences

Code	Species	Accession no.	Ref.
	Phylum Dinozoa, class Dinoflagellatea		
Abe	*Amphidinium belauense*	L13719	22
Ata	*Alexandrium tamarense*	X56946	23
Pmi	*Prorocentrum micans*	M14649	24
Ssp	*Symbiodinium* sp.	M88509	25
Smi	*Symbiodinium microadriaticum*	M88521	25
	Phylum Ciliophora, class Oligohymenophorea		
Cca	*Colpidium campylum*	X56532	26
Eae	*Euplotes aediculatus*	X03949	27
Gch	*Glaucoma chattoni*	X56533	26
Ohe	*Opisthonecta henneguyi*	X56531	26
Pte	*Paramecium tetraurelia*	X03772	28

Phylogenetic relationships are inferred by two methods: (*i*) neighbor joining (NJ) (31), with Tamura's three-parameter distance (32). G+C content in these genes ranges from 35% to 50%; we use the averages for G+C content and transition/transversion ratio. We calculate distances for all substitutions as well as for transversions only. Tree reliability is assessed by the bootstrap method (33) with 1000 replications. All NJ analyses are performed using the program MEGA version 1.0 (34). The genetic distances as well as the sequence alignment are available from the authors upon request.

(*ii*) Maximum likelihood (ML) (35) assumes specific transition/transversion ratios. We use seven transition/transversion ratios (from 1 to 15), which yield similar results. Tree topologies are compared as described (36). Analyses are performed with the algorithm DNAML of the PHYLIP package, version 3.5c (ref. 37; program available from J. Felsenstein, Department of Genetics, University of Washington, Seattle).

We estimate time of divergence by using two rates of nucleotide substitutions: 2% (38, 39) and 0.85% per 100 million years (My). We have obtained the 0.85% rate by comparing the SSU rRNA genes of several sets of increasingly divergent multicellular (mostly metazoan) organisms, for which approximate times of divergence are known.

RESULTS

The NJ trees are shown in Figs. 1 (based on all substitutions) and 2 (transversions only). The five *Plasmodium* species form a monophyletic clade in both trees, with full statistical reliability (bootstrap value, 100%). The nine piroplasm species also form a monophyletic clade (99% and 98% bootstrap).

The *Plasmodium* and piroplasm clades (two distinct orders within the class Hematozoea) are sister clades in Fig. 2, but without statistical reliability (bootstrap, 66%; 70% is a commonly used boundary for statistical significance; ref. 40). In Fig. 1, the piroplasms appear as the sister clade to three coccidian species (*Neospora caninum, Sarcocystis gigantea*, and *Toxoplasma gondii*, family Sarcocystidae) but also without statistical reliability (60%).

The three Sarcocystidae species (*N. caninum, S. gigantea*, and *T. gondii*) form a monophyletic clade in both figures, and so do the two other coccidians (*Cryptosporidium parvum* and *Cryptosporidium wrairi*, family Cryptosporidae), but the class Coccidea is not a monophyletic clade.

The phylum Apicomplexa forms a monophyletic clade, if *Perkinsus* is excluded, but the clade is statistically valid only when transversions alone are used (82%, Fig. 2; bootstrap for this clade in Fig. 1 is 51%). The anomalous genus *Perkinsus* is included within the dinoflagellate clade (79% and 74% in Figs. 1 and 2, respectively). The taxonomic and phylogenetic position of *Perkinsus* has been a matter of uncertainty and debate (1, 2, 18, 21, 29, 41). The dinoflagellate species (phylum Dinozoa), with *Perkinsus* included, form a monophyletic clade, and so do the ciliates (phylum Ciliophora).

We have attempted to resolve the ambiguous NJ phylogenetic relationships within the Apicomplexa phylum by ML methods (data not shown). The tree that associates *Plasmodium* and the piroplasms as sister clades (Fig. 2) is the best ML tree under all transition/transversion values. But this tree is

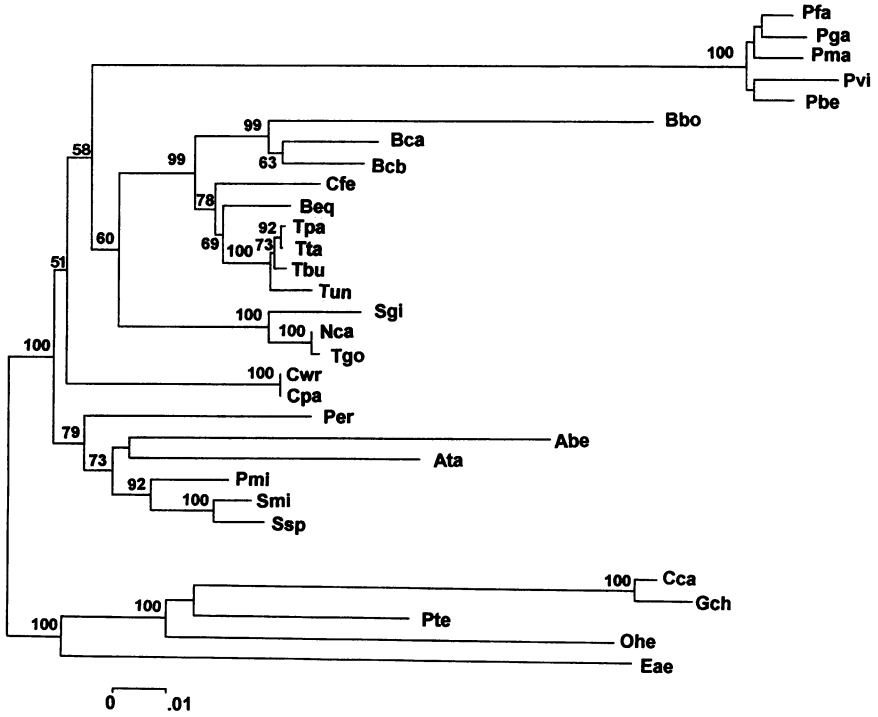

FIG. 1. Phylogenetic relationships among 30 protistan species based on all substitutions. See Tables 1 and 2 for abbreviations of species names. Bootstrap values are shown on the branches; some values below 60 are omitted for clarity.

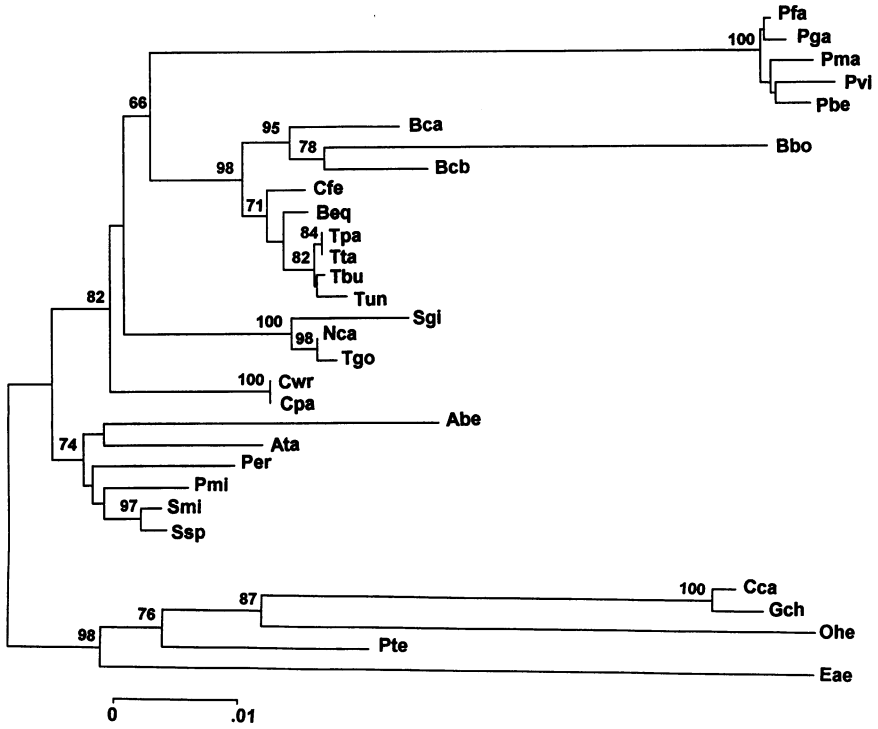

FIG. 2. Phylogenetic relationships among 30 protistan species based on transversions. See Tables 1 and 2 for abbreviations.

not statistically different from trees that show the combination of piroplasms and the coccidian Sasrcocystidae as the sister clade of *Plasmodium* (Fig. 1) or the association of *Plasmodium* and the coccidian Cryptosporidae as the sister clade to the combination of piroplasms and Sarcocystidae. A tree that associates coccidians with piroplasms, and this clade with *Plasmodium*, is significantly worse than the best tree (only for transition/transversion ratios of 10 or higher).

The phylogenetic affinity of *Perkinsus* with the dinoflagellates is confirmed by the ML method. Any monophyletic tree that includes *Perkinsus* with the other Apicomplexa is statistically worse than trees placing *Perkinsus* outside the Apicomplexa. The exclusion of *Perkinsus* (and the class Perkinsidea, which comprises only another genus, *Colpodella*; ref. 2) from the Apicomplexa conforms this phylum with the older phylum Sporozoa (19), a name often treated as a synonym of Apicomplexa (ref. 2, p. 17).

DISCUSSION

The evolution of *Plasmodium* and other Apicomplexa is of particular interest because of the health and economic significance of these parasites. Common views are that the Apicomplexa evolved from parasitic organisms of marine invertebrates (1, 20, 21); that *Plasmodium* evolved as a daughter clade from the coccidians; and that the piroplasms are derived from, or at least are closely related to, *Plasmodium* (1, 20, 21). Some of these views have been based on the postulate that "the probable evolution of the Apicomplexa may be deduced from that of the hosts," which leads to the conclusion, for example, that the gregarines, which are exclusively parasitic on invertebrates, "surely evolved earlier than the other apicomplexan classes, which parasitize vertebrates" (ref. 3, p. 571).

There is no fossil record of apicomplexan organisms (42), but time estimates of their evolution can be obtained by means of a molecular clock. Two conditions need to be met: (*i*) the rate of evolution of the particular gene is constant through time for all lineages—i.e., there is a reliable clock; and (*ii*) the time rate of the clock is known, or alternatively the clock can be calibrated by associating one or more points in a phylogeny with precise times or geological events.

The substitution rate of SSU rRNA genes has been estimated to be 2% per 100 My (38, 39), although Moran *et al.* (43) give a less precise rate of 2–4% per 100 My. One reservation against accepting at face value any one particular rate for the SSU rRNA gene is that the set of nucleotides compared varies from one study to another. Typically, as the array of lineages compared becomes more ancient, and hence more diverse, it becomes increasingly difficult to align the more variable regions of the gene, so that only more conserved regions are included in the comparisons. The estimated rate of evolution decreases accordingly. This effect has been noted by Escalante and Ayala (44): the genetic distances obtained for the rRNA genes of *Plasmodium* species are 2–3 times larger when only *Plasmodium* sequences are aligned than when other Apicomplexa genera are included.

We have determined a rate applicable to our data as follows. We have selected sets of pairwise comparisons among SSU rRNA gene sequences from increasingly divergent multicellular organisms for which reasonably accurate times of divergence are known. The resulting alignment includes 1571 base pairs, close to the 1550 sites aligned for the 30 protistan sequences. The regression of nucleotide substitutions on time yields a rate of 0.85% substitutions per site per 100 My (Fig. 3).

It is apparent in Figs. 1 and 2 that the rate of substitution of the rRNA genes is not constant across all lineages. However, we are primarily interested in the nodes shown in the simplified phylogeny of Fig. 4, but not even the branch averages yield equal substitution rates. The *Plasmodium* and ciliate lineages evolve faster than the piroplasms, coccidians, or dinoflagellates. The issue is which of the Apicomplexa substitution rates is most similar to the rate estimated for the multicellular organisms (Fig. 3).

We deal with the erratic behavior of the protistan molecular "clock" by making the following decisions (Table 3). The length of the branches from nod 1 (Fig. 4) is simply the average observed (Fig. 1) for the *Plasmodium* branches. The length of the branches from node 2 is the average of the *Plasmodium*

lineage (0.094) and the average of the piroplasm and coccidian lineages (0.046). For node 3, we use the average of the *Plasmodium* to dinoflagellate rate (0.103) and the coccidian and piroplasm to dinoflagellate (0.055). For node 4, we obtain the average of the *Plasmodium* to ciliate rate (0.133) and between ciliates and dinoflagellates, coccidians, or piroplasms (0.090). Times are obtained by using our estimated rate of 0.85% substitutions per site per 100 My; Table 3 also gives for comparison the times obtained with the 2% rate (38, 39).

The Coccidea include species parasitic to molluscs and marine annelids, but many are parasitic to mammals and other vertebrates (3). Many coccidians, such as *Cryptosporidium*, are monogenetic parasites, able to complete their life cycle within one single host (the "definitive" host; Table 1). But other coccidians, including *Neospora*, *Sarcocystis*, and *Toxoplasma*, are digenetic (heteroxenous): their complete development requires two successive hosts. Table 3 gives 824 My for the Apicomplexa radiation and thus for the age of the Coccidea. Their old age implies that if they were parasites from the start,

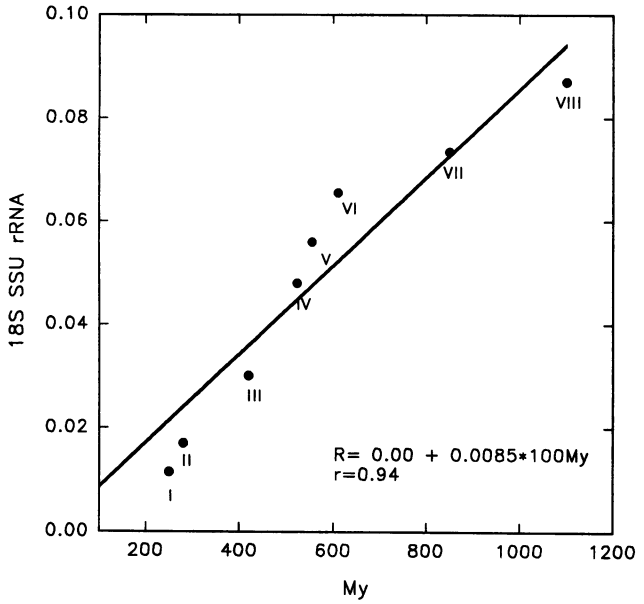

FIG. 3. Rate of evolution of the SSU rRNA genes estimated at 0.85 $\times 10^{-10}$ substitution per site per year (transitions and transversions). Species included and GenBank accession nos. are as follows. Mammals: *Homo sapiens*, X03205; *Mus musculus*, X00686. Reptiles: *Alligator mississippiensis*, M59389; *Heterodon platyrhinos*, M59392. Birds: *Gallus gallus*, M59389; *Turdus migratorius*, M59402. Amphibians: *Bufo valliceps*, M59386; *Xenopus laevis*, K01373. Bony fish: *Fundulus heteroclitus*, M91180. Cartilaginous fish: *Squalus acanthias*, M91179. Agnatha: *Lampetra aepyptera*, M97573. Cephalochordate: *Branchiostoma floridae*, M97571. Arthropods, Insecta: *Acyrthosiphon pisum*, X62623; *Tenebrio molitor*, X07801. Arthropods, Chelicerata: *Eurypelma californica*, X13457; *Amblyomma americanum*, M60487. Molluscs: *Placopecten magellanicus*, X53899; *Limicolaria kambeul*, X66374. Flatworm: *Echinostoma caproni*, L06567. Nematoda: *Haemonchus contortus*, L04153. Jellyfish: *Anemonia sulcata*, X53498. Sponge: *Microciona prolifera*, L10825. Plants: *Zea mays*, K02202; *Alnus glutinosa*, X54984. Fungi: *Aspergillus fumigatus*, M55626; *Dipodascopsis uninucleata*, U00969; *Saccharomyces cerevisiae*, J01353. Divergence times for these organisms are averages of the estimates (45–47). Ordinate values are half the pairwise genetic distances. Rate of substitution is the regression of genetic distance on time when setting the origin at 0, which gives a fit of $r = 0.94$. Without the restriction that it starts at 0, the regression is $-0.697 + 0.009 \times 100$ My, with $r = 0.95$. Roman numerals refer to the following comparisons: I, mammals vs. reptiles vs. birds; II, amphibians vs. lineage I; III, agnatha (lamprey) vs. vertebrates; IV, insects vs. chelicerata; V, protostomes (arthropods and molluscs) vs. lineage III; VI, acoelomates (flatworm) and pseudocoelomates (nematode) vs. lineage V; VII, porifera (sponge) vs. radiata (jellyfish) and lineage VI; VIII, plants vs. fungi vs. animals.

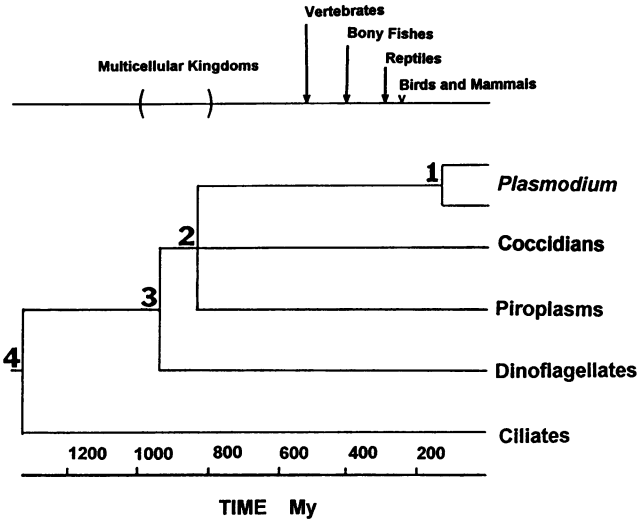

FIG. 4. Simplified phylogeny of Apicomplexa, Dinozoa, and Ciliophora. Scale above highlights the time of origin (first appearance of fossils) of vertebrate groups. Uncertain origin of the animal, plant, and fungi kingdoms is indicated by parentheses.

the coccideans' early hosts could not have been chordates or land organisms because these had not yet evolved. Even if we take 350 My (the lower estimate in Table 3) as the origin of the Coccidea, they would have existed >200 My before the origin of the modern mammal orders (such as Artiodactyla, Carnivora, and Primates) parasitized by coccidians.

The Haemosporida (the order to which *Plasmodium* belongs) and the Piroplasmida are digenetic parasites: maturation of gametes, fertilization, and sporogony occur in the hematophagous invertebrate vector; the rest of the life cycle is completed in the blood of a vertebrate. It is plausible that the monogenetic way of life was the primitive one, but the time frame indicates that vertebrates most likely were not the original hosts. The current digenetic combination of an arthropod (vector) and a vertebrate evolved relatively late in the history of these lineages.

The issue remains as to which lineage, the vector's or the intermediate host's, represents the original monogenetic host. The adaptation of *Plasmodium* and piroplasms to hemotrophy is often considered a derived character (3, 6, 8). However, some authors have proposed that the association of the Hematozoea with vertebrates is ancestral (1, 4, 21, 48). The transmission by arthropod vectors and the origin of complex life cycles would have been, accordingly, a parasite adaptation that evolved because it favors the parasites' rate of transmission (49, 50). The phylogenetic relationships provide no definitive clue for resolving this question. The *Plasmodium* lineage predates the origin of the vertebrates. It is, therefore, possible that the *Plasmodium* lineage evolved as a parasite in parallel with the lineage that eventually gave rise to the vertebrates. The divergence of lizards, mammals, and birds

Table 3. Branch length and time of divergence

Node	Event	Branch length*	Divergence time, My 0.85†	Divergence time, My 2.0†
1	*Plasmodium* radiation	0.011	129	55
2	Apicomplexa radiation	0.070	824	350
3	Origin of Apicomplexa	0.079	929	395
4	Origin of the clade Apicomplexa/Dinozoa	0.112	1317	560

*Branch length is half the average genetic distance.
†Rates in units of 10^{-10} substitution per site per year.

dates from the Triassic, 190–225 My ago (51), before the radiation of the *Plasmodium* genus. Fossil dipterans occur in the Triassic and increase in diversity and abundance from the mid-Jurassic through the Cenozoic (52, 53). The radiation of *Plasmodium*, which we have estimated at 129 My (Table 3), is approximately coincident with the diversification of their vectors' lineages. In any case, the molecular evidence indicates that lateral transfers (i.e, new associations of parasite–vector and parasite–vertebrate host) have occurred in the recent evolution of malaria. The human parasites *Plasmodium falciparum, Plasmodium malariae*, and *Plasmodium vivax* diverged 129 My ago; yet they do not all parasitize the apes (or monkeys), from which the human lineage diverged much more recently. Similarly, these three *Plasmodium* species are transmitted by anopheline vectors of the same or very closely related species, which diverged from each other more recently than the parasites they transmit.

Our results show that digenetic parasitism independently evolved several times in the Apicomplexa as suggested (8). The Apicomplexa radiation predates the origin of the land vertebrates (Table 3); yet digenetic parasitism affecting land vertebrates has evolved in each of the three apicomplexan lineages shown in Fig. 4.

The Apicomplexa/Dinozoa clade may have originated at the time of or earlier than the origin of the multicellular kingdoms. This is consistent with the conclusions of others (29, 54, 55). Thus, the rate of 0.85×10^{-10} substitution per site per year is approximately correct. Paleontological and molecular data indicate that the radiation of red and green algae, ciliates, and dinoflagellates occurred near the Mesoproterozoic–Neoproterozoic boundary (56), consistent with our estimate of 1317 My for the time of divergence of the ciliates from the ancestral lineage of dinoflagellates and Apicomplexa.

We are grateful to Walter Fitch, Richard Hudson, and Anthony James for comments on the manuscript. Work supported by a fellowship from the Banco Interamericano de Desarrollo-Consejo Nacional de Ciencia y Tecnologia, Venezuela (A.A.E.) and National Institutes of Health Grant GM42397 (F.J.A.).

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