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RESEARCH ARTICLE

Evolutionary Relatedness of *Plasmodium* Species as Determined by the Structure of DNA

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Malaria parasites are classified in the genus *Plasmodium*. Historically, the species were grouped according to the hosts that they infected and then were subdivided according to morphological and biological characters (*1, 2*). Thus *Plasmodium* species are classified into primate, rodent, avian, and lizard malarias. The implication is that the parasites have evolved with their hosts and that there is greater relatedness among parasites in related hosts than those in hosts greatly separated in evolution. In the present article we examine this hypothesis using analysis of base composition and organization of DNA from various species of *Plasmodium*. We find a major exception to the classically derived organization of the malarial evolutionary tree. Surprisingly, *P. falciparum*, the malaria of man that causes the most morbidity and mortality, appears more closely related to rodent and avian malarias than to other primate malarias. Further, our data allow us to suggest which common characteristics of different *Plasmodium* species are the result of direct inheritance and which may be the result of independently occurring but convergent events.

Analysis of Base Composition

The deoxyguanosine · deoxycytidine (dG · dC) content of DNA from *P. falciparum* (*3*) and *P. berghei* (*4*) (18 and 24 percent, respectively) differs greatly from that of the mammalian host (~37 percent). We purified DNA samples from *P. falciparum, P. knowlesi*, and *P. berghei*, tested them for host DNA contamination, and then analyzed them for total dG · dC content by a DNA duplex melt procedure. Purification of parasite DNA is critical for these experiments since one nucleated host cell per 100 parasites would result in approximately a 50 percent contamination due to differences in total genome size. Each preparation of parasite DNA was tested for purity as described (see legend to Fig. 1). *Plasmodium falciparum* DNA that had been analyzed previously (*3*) was used as a control. *Plasmodium falciparum* parasites in continuous culture in human erythrocytes in vitro (*5*) were nearly free of nucleated host cells. The *P. falciparum* DNA was radiolabeled by nick translation with either deoxyadenosine triphosphate or deoxycytidine triphosphate and the dG · dC content was determined. The melting temperature (T_m) of both deoxyadenosine- and deoxycytidine-labeled DNA indicated a dG · dC content of 18 percent, consistent with the published figure (*3*). *Plasmodium berghei* DNA was extracted from infected mouse erythrocytes (*6*) and was further purified by $Hg^{2+}CsSO_4$ or Hoechst dye CsCl gradients as shown below. The dG · dC content of the *P. berghei* DNA was found to be 18 percent. It had previously been reported to be 24 percent (*4*). The difference probably can be attributed to host DNA contamination in the previous study. Determination of the dG · dC content of *P. knowlesi*, a malaria

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Abstract. *Malaria parasites can be grouped evolutionarily by analysis of DNA composition and genome arrangement. Those that vary widely with regard to host range, morphology, and biological characteristics fit into only a small number of distinctive groups. The DNA of the human parasite* Plasmodium falciparum *fits into a group that includes rodent and avian malarias and is unlike the DNA of other primate malaria parasites. The DNA of* Plasmodium vivax, *which is also a human parasite, fits into a distinctly different group that includes* Plasmodium cynomolgi, *a parasite of monkeys. The evolutionary lines suggested here appear to be consistent with similarities seen among malaria parasites with regard to gene sequence.*

of monkeys, was more difficult, since it was not possible to separate its DNA from host DNA by Hoechst dye CsCl gradients. We made several preparations of *P. knowlesi* parasites in an attempt to eliminate nucleated host cells from the preparation. DNA from one of these preparations was tested and shown to contain no host DNA (Fig. 1A). Its dG · dC content was 30 percent (Fig. 1B).

Hoechst dye CsCl centrifugation is known to be a useful procedure to separate DNA's of different dG · dC content (*7, 8*). Here we show that the position of banding in the Hoechst dye CsCl gradient can be correlated with dG · dC content. *Plasmodium falciparum* (Fig. 2A) was shown to contain one density band. This preparation had no visible band of contaminating host DNA. In separate experiments (*8*) we have shown that this band contains all the *P. falciparum* DNA that is metabolically labeled in vivo with hypoxanthine. In the present experiments, DNA from *P. berghei*–infected blood (Fig. 2B) had two separated components. Previously we found that the top band contained only parasite DNA and that the bottom band contained mouse DNA (*8*). In the present experiments, *P. knowlesi* DNA was not separable from monkey DNA. Only one broad band appeared on the gradient and subsequent analysis showed that it hybridized to both parasite and monkey DNA sequences (Figs. 2E and 3). This is consistent with our determination that the dG · dC content of *P. knowlesi* DNA is 30 percent. Thus for these three *Plasmodium* species, the density at which the parasite DNA's banded in CsCl centrifugation was consistent with the dG · dC content.

Samples of DNA from several other primate *Plasmodium* species were tested by Hoechst dye CsCl gradients to see if they were similar in density to *P. berghei* and *P. falciparum* or to *P. knowlesi*. Density in CsCl was used as a rough measure of dG · dC content. DNA's of different densities were analyzed for host and parasite DNA by Southern blot analysis and hybridization to radiolabeled monkey DNA or to cloned ribosomal genes from *P. berghei* (*9*) or a purified fraction of parasite DNA. DNA from *P. vinckei* and *P. chabaudi*, two rodent malaria parasites, was isolated free of host DNA and banded as a single low-density component at the same place as the low-density parasite band of *P. berghei*. *Plasmodium lophurae*, an avian parasite, is composed of DNA that bands at the CsCl density similar to that of the low dG · dC content of *P. falciparum* and rodent malarias. A sample of *P. fragile* DNA was tested because this parasite is thought to be the monkey equivalent to the human parasite *P. falciparum* (*2*). The DNA of *P. fragile*, however, has a dG · dC content more closely aligned with that of *P. knowlesi* DNA than with that of *P. falciparum* (Fig. 2F). When the single band of DNA from the *P. fragile* gradient was tested by Southern blot analysis, the hybridization patterns showed the presence of both parasite (Fig. 3) and monkey DNA (data not shown).

We also tested DNA from *P. cynomolgi*, a monkey parasite, because, unlike the aforementioned parasites, it causes a relapsing malaria, that is, latent parasites in the liver cause repeated clinical attacks. It is similar to *P. vivax*, which causes a relapsing malaria in humans. *Plasmodium cynomolgi* DNA is composed of both high- and low-density components and of multiple minor bands (Fig. 2G). The total low-density fraction (refractive index = 1.3936) was radiolabeled and used to probe Hind III restricted DNA from *P. knowlesi*, which does not have a low-density component, and from the high dG · dC component of *P.*

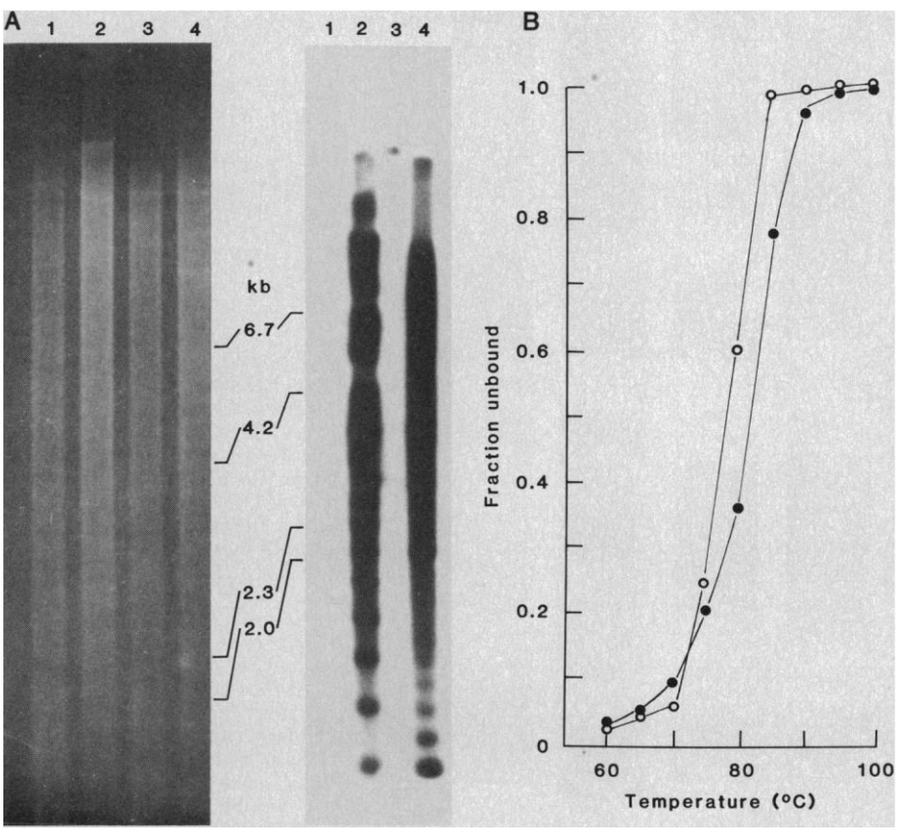


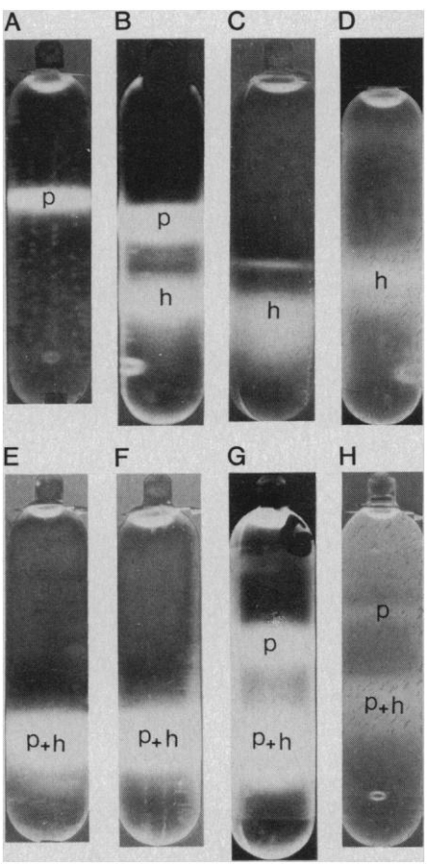
Fig. 1. (A) DNA isolated from a preparation of *P. knowlesi* is free of contamination with monkey DNA. Restricted DNA (2.0 μg) from *P. knowlesi* (lanes 1 and 3) and rhesus monkey (lanes 2 and 4) was subjected to electrophoresis through agarose, stained with ethidium bromide (left) and the Southern blot hybridized with radiolabeled monkey DNA (right). Lanes 1 and 2 contain DNA restricted with Eco RI. Lanes 3 and 4 contain DNA restricted with Hind III. (B) *Plasmodium knowlesi* DNA and *P. berghei* DNA contain 30 and 18 percent dG · dC, respectively. Parasite DNA was labeled with ^{32}P by nick translation and then sheared to a length of 300 to 500 base pairs in a VirTis homogenizer. Melting experiments were done in 0.12*M* sodium phosphate buffer and the dG · dC content was determined as described by Marmur and Doty (*17*). Symbols: ○, *P. berghei* DNA (T_m, 77°C); ●, *P. knowlesi* DNA (T_m, 81°C).

cynomolgi. Only two homologous bands were found in the high-density component of *P. cynomolgi* DNA but over 20 in the *P. knowlesi* DNA. All bands were specific for parasite DNA (data not shown). Thus certain DNA sequences in *P. knowlesi* DNA appear to be partitioned into high- and low-density components in *P. cynomolgi*. The high-density component of *P. cynomolgi* has all but one of the parasite ribosomal genes (Fig. 3) as well as other sequences homologous to the foreign probes described in Table 1. *Plasmodium vivax* DNA from infected squirrel monkeys was arranged as *P. cynomolgi* DNA. The low-density band of *P. vivax* hybridized with the low-density band of *P. cynomolgi* while the ribosomal genes and other probes hybridized with the high-density band.

Table 1. The relation between dG · dC content and hybridization with gene probes for various malarial DNA's. The dG · dC content of *P. falciparum*, *P. berghei*, and *P. knowlesi* were determined as described in Fig. 1. The dG · dC content of the DNA of the other parasites was estimated by the density at which it banded in Hoechst dye CsCl gradients (Fig. 2). Southern blots of parasite DNA's were done as described for thymidylate synthetase in Fig. 4. Symbols: +, present; −, absent; DHFR, dihydrofolate reductase; TS, thymidylate synthetase; N.D., not done.

Species	Host	dG · dC content		Gene hybridization			
		18 percent	~30 percent	Actin	Tubulin	DHFR	TS
P. falciparum	Man	+	−	−	−	−	+
P. berghei	Rodent	+	−	−	−	−	+
P. lophurae	Bird	+	−	−	−	−	+
P. knowlesi	Monkey	−	+	+	+	+	−
P. fragile	Monkey	−	+	+	+	+	−
P. cynomolgi	Monkey	+*	+	+	+	+	−
P. vivax	Man	+*	+	+	+	N.D.	N.D.

*Multiple minor bands in addition to the two major bands at 18 percent and ~30 percent dG · dC content.



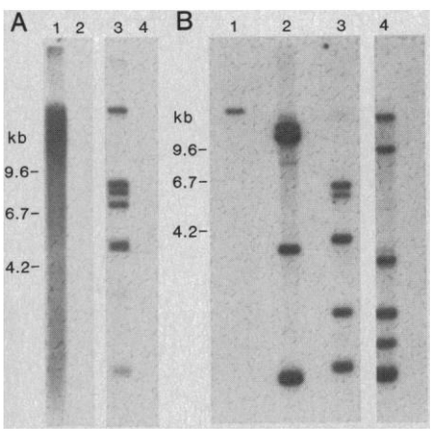


Fig. 2 (left). The density bands formed by DNA of different *Plasmodium* species after Hoechst dye CsCl centrifugation (p, parasite; h, host). Between 5 and 20 μg of DNA from each organism was bound to an equal weight of Hoechst dye 33258 and then centrifuged in a CsCl solution (refractive index of 1.3950) as described (*8*). DNA was visualized by ultraviolet illumination. The CsCl containing the low-density band had a refractive index of 1.3936 (density of 1.6369 per cubic centimeter). The CsCl solution containing the broad, higher density band had a refractive index of 1.3972. (A) *Plasmodium falciparum* DNA isolated from culture; (B) *P. berghei* DNA from infected erythrocytes of mice; (C) mouse DNA; (D) rhesus monkey DNA; (E) *P. fragile* DNA; (F) *P. knowlesi* DNA; (G) *P. cynomolgi* DNA from infected erythrocytes of rhesus monkeys; and (H) *P. vivax* DNA from infected erythrocytes of squirrel monkeys. Fig. 3 (right). (A) *Plasmodium falciparum* DNA isolated from an *Aotus* monkey was separated from host DNA in a Hoechst dye CsCl gradient like those shown in Fig. 2. Two bands were separated and subsequently analyzed by restriction and Southern blot analysis. Southern blots of equal amounts of DNA (1 μg) from the top (lanes 2 and 3) and bottom bands (lanes 1 and 4) hybridized to either radiolabeled monkey DNA (left panel), or radiolabeled plasmid (pPb5.6) containing a *P. berghei* ribosomal gene (*9*) (right panel). (B) DNA from the high-density CsCl bands (refractive index of 1.3972) shown in Fig. 2, E, F, and G, were isolated and subjected to Hind III restriction and Southern blot analysis. The blot was hybridized to the radiolabeled *P. berghei* ribosomal gene probe described above. Lane 1, rhesus DNA; lane 2, *P. knowlesi* DNA; lane 3, *P. fragile* DNA; lane 4, *P. cynomolgi* DNA from the lower band in Fig. 2B.

Hybridization Experiments

From the analysis of total base composition we conclude that *P. falciparum* is more related in evolution to rodent and avian malaria parasites than to other primate malaria parasites. If this is the case, genes coding for homologous proteins of *P. falciparum* should show greater relatedness to rodent and avian malarias than to the other primate malarias. Ideally this assumption would be tested by cloning and sequencing multiple genes from each system. However, we tried to establish a general trend by another approach. DNA clones for chicken and *Dictyostelium* actin (*10, 11*), *Chlamydomonas* tubulin (*12*), mouse dihydrofolate reductase (*13*), and yeast thymidylate synthetase (*14*) were radiolabeled and hybridized with parasite genomic DNA by the Southern blot technique. The stringency of blot washing was increased until hybridization was restricted to only one or two parasite DNA's. In this manner it was determined which parasite DNA's contained the sequences most closely related to the various probes (Fig. 4 and Table 1). Thymidylate synthetase hybridized only to *P. falciparum*, *P. berghei*, and *P. lophurae* and not to *P. knowlesi* and *P. cynomolgi*. Conversely, a complementary DNA (cDNA) clone of dihydrofolate reductase, chicken actin, and tubulin hybridized to *P. knowlesi* and *P. cynomolgi* and not to *P. falciparum*, *P. berghei*, and *P. lophurae* under the conditions described (see Fig. 4). A *Dictyostelium* actin clone hybridized to DNA from all the parasites under our conditions.

Conclusions

Malaria parasites were previously grouped primarily with regard to host range and secondarily with regard to morphology and biological characters (*1, 2*). By comparative DNA analysis, we have found relationships that contradict these criteria. Thus, *P. falciparum*, *P.*

berghei, and *P. lophurae* show similarities in DNA (Table 1) despite differences in host range (man, mouse, and bird, respectively), morphology (for example, sausage-shaped gametocytes of *P. falciparum* and round gametocytes of *P. berghei*), and biology (for example, periodicity varying from 24 to 48 hours). *Plasmodium falciparum* appears less related to other primate malaria parasites including *P. fragile* which, like *P. falciparum*, undergoes deep vascular schizogony. Erythrocytes containing mature asexual parasites of *P. falciparum* and *P. fragile* are rarely seen in the peripheral blood because they bind to endothelium via "knobs," protrusions on the erythrocyte surface (*15*). We suggest that these similarities in structure and function are examples of convergent evolution and do not reflect the closeness of heritage. Other similarities in structure [for example, the gametocytes (*16*) of *P. falciparum* and avian parasites] may indeed reflect evolutionary relatedness.

On the basis of the similarity of their DNA's, we suggest that *P. knowlesi* and *P. fragile* are related despite the differences in periodicity of their asexual erythrocytic stages (24 and 48 hours, respectively) and the absence of knobs and sequestration in *P. knowlesi*. It is interesting that the distribution of both parasites is limited by the distribution of the *Anopheles leucosphyrus* group of mosquitoes in Sri Lanka (*P. fragile*) and Malaysia (*P. knowlesi*) (*2*).

We have confirmed the similarities of *P. vivax* in man and *P. cynomolgi* in the monkey. Both cause relapses, with repeated attacks of malaria resulting from latent forms of the parasite in hepatic parenchymal cells, and both have pink stippling of the infected erythrocyte in Giemsa stain (Schüffner's dots). Both parasites produce multiple banding of DNA on CsCl gradients with two major bands. The gene probes hybridize with *P. cynomolgi* DNA in the *P. knowlesi* pattern and all hybridization is with DNA from the high-density band (~30 percent dG · dC content). The low-density DNA of *P. cynomolgi* appears to have more homology to the DNA of *P. knowlesi* than to the high-density DNA of *P. cynomolgi*, suggesting a separation of genes. We speculate that the genes in this fraction may be responsible for a specific stage in the life cycle (for example, relapses or gametocytes). Furthermore, one can speculate that a shifting of genes to one density component or another facilitated speciation.

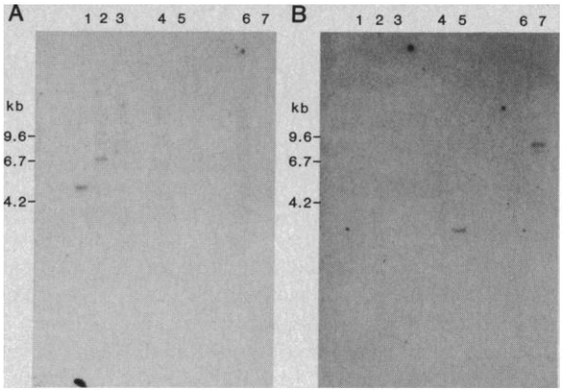


Fig. 4. DNA from several different parasites were compared with regard to their degree of homology with a cDNA clone of actin from chicken (*10*) and a clone of thymidylate synthetase from yeast (*14*). Two micrograms of DNA from each parasite and host was restricted with Hind III and analyzed by Southern blot analysis. (A) The blot was hybridized with a radiolabeled actin probe and washed in 0.1 × SSC (standard saline citrate) and 0.1 percent (weight to volume) sodium dodecyl sulfate for 1 hour at 50°C with five solution changes. (B) The blot was hybridized with a radiolabeled thymidylate synthetase probe and washed in 0.2 × SSC and 0.1 percent (weight to volume) sodium dodecyl sulfate for 1 hour at 50°C with five solution changes. Lane 1, *P. fragile* DNA; lane 2, *P. knowlesi* DNA; lane 3, rhesus monkey DNA; lane 4, mouse DNA; lane 5, *P. berghei* DNA; lane 6, human DNA; and lane 7, *P. falciparum* DNA.

One implication of the present study is that homologous proteins and their genes should be more similar within a group than between groups. This seems to be the case at least when tested with respect to measuring the various degrees of homology of parasite DNA to cloned gene probes from other organisms. However, these results are also consistent with the possibility that different *Plasmodium* species independently acquired a dG · dC content of either 18 percent or 30 percent and that these contents were reflected in the gene makeup. Since we are measuring similarities among members of a single genus, we think that this is less likely and that the sequence relatedness is a measure of genetic relatedness.

The biology of *Plasmodium* has been studied extensively. A great number of characteristics have been examined and categorized in order to relate the different *Plasmodium* species. We suggest that these same species can be separated into a small number of evolutionarily related groups simply on the basis of their DNA composition. The parasite DNA's that we have tested to date fall into one of three categories: (i) a single component DNA containing 18 percent dG · dC, (ii) a single component DNA containing 30 percent dG · dC, or (iii) a genome containing both high- and low-density components. It will be interesting to see which of the biological characteristics of *Plasmodium* fit directly into this scheme of evolution and which characteristics seem to be the result of a convergent evolution perhaps resulting from the pressures caused by the host immune response.

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18. We thank I. Green and F. Eden for suggestions on the manuscript.

21 March 1984; accepted 4 May 1984