Title
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Permalink
https://escholarship.org/uc/item/31v5p8p3

Journal
Proceedings of the National Academy of Sciences of the United States of America, 106(35)

ISSN
0027-8424

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Publication Date
2009-09-01

DOI
10.1073/pnas.0907740106

Supplemental Material
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Peer reviewed
The origin of malignant malaria

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Contributed by Francisco J. Ayala, July 13, 2009 (sent for review June 29, 2009)

Plasmodium falciparum, the causative agent of malignant malaria, is among the most severe human infectious diseases. The closest known relative of P. falciparum is a chimpanzee parasite, Plasmodium reichenowi, of which only one single isolate was previously known. The co-speciation hypothesis suggests that both parasites evolved separately from a common ancestor over the last 5–7 million years, in parallel with the divergence of their hosts, the hominin and chimpanzee lineages. Genetic analysis of eight new isolates of P. reichenowi, from wild and wild-born captive chimpanzees in Cameroon and Côte d’Ivoire, shows that P. reichenowi is a geographically widespread and genetically diverse chimpanzee parasite. The genetic lineage comprising the totality of global P. falciparum is fully included within the much broader genetic diversity of P. reichenowi. This finding is inconsistent with the co-speciation hypothesis. Phylogenetic analysis indicates that all extant P. falciparum populations originated from P. reichenowi, likely by a single host transfer, which may have occurred as early as 2–3 million years ago, or as recently as 10,000 years ago. The evolutionary history of this relationship may be explained by two critical genetic mutations. First, inactivation of the CMAH gene in the human lineage rendered human ancestors unable to generate the sialic acid Neu5Gc from its precursor Neu5Ac, and likely made humans resistant to P. reichenowi. More recently, mutations in the dominant invasion receptor EBA 175 in the P. falciparum lineage provided the parasite with preference for the overabundant Neu5Ac precursor, accounting for its extreme human pathogenicity.

Malaria counts among the worst scourges of humankind, accounting for some 500 million clinical cases per year and more than one million deaths, mostly children (1). It amounts to an immeasurable health burden and inhibits economic prosperity in numerous tropical countries, most extensively in Africa. Plasmodium falciparum is the most virulent among the four Plasmodium species parasitic to humans, accounting for ~85% of all malaria cases, and nearly all of the mortality. The extreme pathogenicity of P. falciparum has suggested that it is a recent human parasite, acquired by transfer from a nonhuman host (2). Some early molecular phylogenies seemed to be consistent with this hypothesis, because they showed P. falciparum to be more closely related to Plasmodium gallinaceum, a chicken parasite, than to any of the other human parasite species (3). A considered possibility was that P. falciparum evolved from an avian parasite following a horizontal host transfer, perhaps in association with the Neolithic domestication of the chicken. It was, however, shown by Escalante and Ayala (4) and Escalante et al. (5) that the closest relative of P. falciparum is P. reichenowi, a malaria parasite isolated from a captive chimpanzee that had not been included in earlier studies. These authors showed that P. falciparum and P. reichenowi form an independent clade distinct from other malaria parasites, including the other three human malaria parasites, which appear to have originated in Old World monkeys (4, 5). The close phylogenetic relationship between P. falciparum and P. reichenowi, their distinctness from the other human malaria parasites, and their remoteness from bird or lizard parasites was soon confirmed by other studies (6–8).

Three mutually exclusive hypotheses could account for the relationship between P. falciparum and P. reichenowi. (i) Co-speciation: P. falciparum and P. reichenowi evolved from a common ancestor parasite, independently in their respective hosts, humans and chimpanzees, over the last 5–7 million years; (ii) Human origin: P. reichenowi evolved from an introduction of P. falciparum into chimpanzee hosts; or (iii) Chimpanzee origin: P. falciparum evolved from the introduction of the chimpanzee P. reichenowi into the human lineage (Fig. 1).

These hypotheses can readily be tested by determining the phylogenetic relationships among the two parasite species and by comparing their levels of genetic polymorphism, particularly with respect to silent nucleotides, which are expected to accumulate as a function of the rate of mutation and the time elapsed since the origin of a species. If co-speciation occurred, P. falciparum and P. reichenowi should form distinct, sister clades. If hypotheses ii or iii are correct, the level of neutral polymorphism should be greater in the ancestral parasite than in the derived species. In the absence of the molecular characterization of multiple strains of P. reichenowi, of which only one strain was available, Escalante and Ayala (4) and Escalante et al. (5) favored the co-speciation hypothesis, as soon did other investigators (6–8). The two alternative transfer hypotheses were largely ignored because of the availability of only one known isolate of P. reichenowi (from Cameroon, described in 1917 and 1920, ref. 9). However, Martin et al. (10) suggested a mechanism compatible with hypothesis iii on the basis of differential expression of host sialic acid (Sia) ligands and differences in parasite receptor Sia-binding preferences.

Rich, Ayala, and collaborators soon demonstrated that P. falciparum has very low levels of neutral polymorphism (11–13), a result that was subsequently confirmed by other investigators (14–18). The scarcity of neutral polymorphisms in P. falciparum was interpreted to be the result of a recent world expansion of...
the species, which was estimated to have happened a few thousand years ago, rather than millions of years ago (the Malaria Eve hypothesis; ref. 13).

The recent world expansion of *P. falciparum* could have come about in two ways. First, as a consequence of a severe bottleneck, so that all extant populations of the species would have derived from a few surviving strains. An alternative, equally viable explanation would be that *falciparum* malaria had been restricted to a local population (somewhere in tropical Africa), from where it would have expanded through much of the African tropics and beyond as a consequence of recent environmental and vector changes (12, 13, 19–21). These changes would include, among others, the introduction of agriculture in tropical Africa during the late Neolithic and consequent deforestation, creating pools of standing water and other conditions favorable for mosquito breeding; the evolution of anthropophilic *Anopheles* vectors (19, 20); and the gradual warming of the planet that eventually allowed the geographic expansion of the vector–parasite association (11, 13, 22). These three sets of events are timed within the past 10,000 years, which is consistent with the estimated time of the world expansion of *P. falciparum* (13, 21, 22). The current availability of additional isolates of *P. reichenowi* makes it now possible to investigate comparatively these hypotheses.

**Results**

We have sampled wild and wild-born captive chimpanzees from Côte d’Ivoire (n = 10) and Cameroon (n = 84). From the tissues collected from these chimpanzees, we have identified eight new isolates of *P. reichenowi* by amplifying three gene fragments that have been extensively used in *Plasmodium* studies: mitochondrial *cytB*, apicoplast *clpC*, and nuclear *18S rRNA*. Five isolates were derived from the 84 Cameroonian chimpanzees and three from the 10 chimpanzees from Côte d’Ivoire. One of the Ivoirian chimpanzees, Rafiki, was infected with two strains of *Plasmodium*, characterized by distinct nucleotide sequences in each of the three gene fragments. Because our blood samples were collected on filter paper, we were unable to determine whether the parasites’ gametocytes were crescent-shaped, an attribute exclusively characteristic of *P. falciparum* and *P. reichenowi* (23).

However, the diagnostic repeat region from the circumsporozoite protein (*Csp*) gene from five sequenced isolates (Bana, Gabon, Max, Nino, and Loukoum) shows that each gene encodes amino acid repeat motifs that specifically distinguish *P. falciparum* and *P. reichenowi* from all other malarial parasites (Fig. 2) (24), consistent with their designation as *P. reichenowi*.

We have examined the nucleotide polymorphisms among the three gene fragments. As reported previously, nucleotide polymorphisms among *P. falciparum* isolates are extremely scarce (18, 21, 25, 26). Among the *P. reichenowi* isolates, there is considerably greater nucleotide variation than in *P. falciparum*, nearly all of which occurs among silent (synonymous) sites in the two amino acid coding regions (*cytB* and *clpC*) (Fig. 3). The number of synonymous differences among sequences is expected to increase when evolutionarily independent lineages are combined. However, the mean pairwise number of nucleotide differences (π) is unaffected when sequences from 133 isolates of *P. falciparum* are added to the nine isolates of *P. reichenowi* (Fig. 3). This observation, coupled with the related observation of much greater diversity among *P. reichenowi* isolates than among *P. falciparum* isolates (even though these are greatly more numerous) is inconsistent with the co-speciation hypothesis and supports the hypothesis that *P. falciparum* evolved from a chimpanzee parasite.

Fig. 4 shows the phylogenetic relationships among the eight new isolates of *P. reichenowi*, plus the one previously reported isolate (CDC1) together with 133 strains of *P. falciparum*, based on the *cytB* gene sequences (see Table S1). We have included in this phylogeny the three other human malaria parasites (*P. vivax*, *P. ovale*, and *P. malariae*), as well as two malaria parasites from African rodents (*P. yoelii* and *P. berghei*), and an avian malaria parasite (*P. gallinaeum*). The *cytB* gene (Fig. 4), which...
is thought to be best for recovering the deeper divergences within the genus (7, 8), well illustrates the results from all three gene fragments (for the clpC and 18S rRNA phylogenies, see Fig. S1 and Fig. S2, respectively). It demonstrates that the genetic diversity of *P. falciparum* is unambiguously nested within the set of the chimpanzee parasites.

The competing hypotheses concerning the origins of *P. falciparum* and *P. reichenowi* (Fig. 1) can also be tested by estimating the evolutionary “cost” imposed by constraining phylogenetic searches to only those trees consistent with co-speciation. This constraint lengthens the most parsimonious trees for the cytB, clpC, and 18S rRNA by 12, 2, and 3 steps, respectively, and reduces the probability of the observed results by 70-fold, 15.8-fold, and 3.6-fold, by using optimized evolutionary models (see Materials and Methods). The evidence against the reciprocal monophyly of *P. falciparum* and *P. reichenowi* (i.e., common ancestral origin; Fig. 1, Left) relative to other phylogenetic alternatives is strong for clpC and decisive for cytB and 18S rRNA (Bayes factors >11, 949, and 910, respectively) (27–29). Evidently, the polymorphism observed in these genes did not accumulate independently in parasite lineages that were restricted to either human or chimpanzee hosts.

**Discussion**

Our results do not support the co-speciation hypothesis and strongly favor hypothesis iii, namely that *P. falciparum* evolved from *P. reichenowi*. Indeed, our data favor five significant conclusions, two that confirm previous results and three new ones: (I) *P. falciparum* and *P. reichenowi* combined are monophyletic (4, 5); (II) *P. falciparum* has very low levels of polymorphism (12–18). Fig. 4 includes 133 strains of *P. falciparum*, representative of the world distribution of the species. Yet, the total variation is extremely small; for example, the terminal branch labeled “*P. falciparum*” includes 96 strains from three different continents, with an average pairwise polymorphism of 0.01 (8). (III) *P. reichenowi* is a geographically widespread and genetically very polymorphic species, with levels of polymorphism greater than the average divergence between the previously known CDC1 strain of *P. reichenowi* and all strains of *P. falciparum* (see Figs. 3 and 4). The overall polymorphism of only nine *P. reichenowi* isolates is much greater than the polymorphism of all 133 *P. falciparum* isolates. (IV) The 133 strains representative of the world populations of *P. falciparum* are all included as one monophyletic branch of the *P. reichenowi* tree. (V) Based on our data, there has likely been only one host transfer from chimpanzee to humans (*P. reichenowi* to *P. falciparum*), shown by the fact that all world strains of *P. falciparum* are connected to a single branch of the *P. reichenowi* tree (see Fig. 4 for cytB and Figs. S1 and S2 for ClpC and 18S rRNA).

Ollomo et al. (30) have recently isolated two strains of *Plasmodium* parasites from two wild-born chimpanzees. The cytB sequences of the two strains are 99.8% identical (out of 866 nucleotides), but they are quite different (91–92%) from the cytB from the previously known *P. reichenowi* strain (CDC1). Ollomo et al. estimate that the divergence between their newly isolated strains and CDC1 may have occurred as early as 21 ± 9 Mya, if the divergence between *P. reichenowi* CDC1 and *P. falciparum* is assumed to be 4–7 million years (Myr) old, consistent with the co-speciation hypothesis and with previous estimates (12, 13, 22). If the divergence between *P. reichenowi* and *P. falciparum* occurred during the last 2.8 Myr, as proposed by Martin et al. (10), the divergence between *P. reichenowi* and the two new *Plasmodium* isolates would have occurred within the last 10 Myr. Ollomo et al. (30) have proposed that the two new strains be considered a new species, named *Plasmodium gaboni*. We have analyzed the cytB sequence of the two new strains in comparison with our newly isolated strains from chimpanzees, and they fit within the clearly distinct clade consisting of three of our strains: Bana, Max, and Loukoum (Fig. 4). Ollomo et al.’s (30) proposal implies that these three strains would belong to the new species, *P. gaboni*. This would be a reasonable inference, which we are for the moment leaving in abeyance until more definitive estimates of the divergence times or other information becomes available.

The distinguished anthropologist Frank B. Livingstone conjectured that *P. falciparum* may have been acquired by a transfer to humans of a chimpanzee parasite (31). The plausibility of Livingstone’s hypothesis was based on the supposition that, as humans developed increasingly larger agricultural societies, they encroached upon the dwindling forest habitats of species such as the chimpanzee, and so there may have been repeated opportunities for horizontal transfer. Today, human encroachment into the last forest habitats has further extended, leading to a higher risk of transfer of new pathogens, including new malaria parasites. Our results confirm Livingstone’s conjecture and, moreover, suggest that the world’s extant populations of *P. falciparum* derive from a single transfer of *P. reichenowi* from chimpanzees to humans.
How and when did the host transfer occur? A hypothesis proposed in the past was that the ancestors of *P. falciparum* would have been transferred from another host to humans as our Neolithic ancestors transitioned from hunter-gatherers to agriculturalists some 10,000 years ago (22, 31). This proposal was based on anthropological information about the history of our species, but also on the estimated age of hemoglobin mutants that render humans resistant to malaria infection. The Malaria Eve hypothesis, based on *P. falciparum*’s very low levels of neutral polymorphisms, is consistent with this hypothesis (13, 21), which is also supported by the recent evolution of anthropophilic vectors and by climatic considerations (19, 20, 22). Coluzzi and colleagues have shown that the rapid incipient speciation of the principal African vectors of *P. falciparum* was driven by postagricultural human conditions, which were “a key influence on the origin of the modern *P. falciparum* from an ancestral, less pathogenic, taxon” (20).

Other investigators subsequently have sought to determine the time of the most recent common ancestor of extant *P. falciparum* populations. A time-period consistent with a postagricultural introduction remains most probable (18, 25). Indirect corroboration of this hypothesis lies within the human genome. Specific genetic mechanisms that have given humans resistance to malaria, such as G6PD deficiency and other hemoglobinopathies, appear to have arisen within a time-frame consistent with a postagricultural origin (16, 17).

Our results confirm that the extant populations of *P. falciparum* have originated in the recent past, because of their very low genetic diversity, but our results do not tell us when the transfer to humans may have occurred. This transfer must have happened much earlier, allowing for the differentiation of *P. falciparum* from *P. reichenowi*, which may have originally consisted of nothing more than a change in binding specificity. At present, the divergence between the two species is not large, although much larger than the divergence among the extant *P. falciparum*. It seems likely that considerable time, on the order of many tens or hundreds of thousands of years, may have elapsed from the time of the host transfer to the time when genetic changes in the parasite and/or the human lineages made possible the rapid expansion of *P. falciparum*.

Martin et al. (19) have suggested that the classic failure of experimental cross-infection of *P. falciparum* into chimpanzees and of *P. reichenowi* into humans can be explained by a human-specific mutation event. Whereas the *P. falciparum* merozoite can use multiple pathways to invade erythrocytes (32), the dominant invasion receptor appears to be the erythrocyte-binding-like (EBA)-175. The underlying polypeptide sequence of its primary target molecule in erythrocytes is the *Sia*-capped N-terminal domain of the major erythrocyte glycoprotein glycoporphin-A (GYP-A), which itself is undergoing rapid evolution (33), presumably due to selection pressure from the parasite.

Several million years after divergence from the chimpanzee lineage, a major biochemical change in Sia biology occurred in the human ancestral lineage (34, 35). The most common Old World primate Sias are N-glycolylneuraminic acid (Neu5Gc) and its metabolic precursor, N-acetylglycolylneuraminic acid (Neu5Ac), which differ by a single oxygen atom. A mutation in the CMAH gene makes humans unable to produce Neu5Gc from Neu5Ac, which accumulates in great excess on human erythrocytes. Chimpanzees carry both Sias, with Neu5Ac being dominant (10, 36). *P. reichenowi* EBA-175 has a marked preference for Neu5Gc, which was suggested to represent the ancestral condition, whereas *P. falciparum* EBA-175 has a preference for Neu5Ac, which accumulates in humans as a consequence of the CMAH mutation (10, 36). Martin et al. (10) thus conjectured that the loss of Neu5Gc in the human lineage (by way of the CMAH mutation) would have provided our emerging *Homo* ancestors, perhaps as early as 2–3 million years ago, with temporary relief from *P. reichenowi* malaria (8, 10). Indeed, it is possible that the CMAH mutation was driven to fixation by continued selection pressure from the then extant form of *P. reichenowi*. The parasitic malignancy of *P. falciparum* would have come about later, by selective evolution of its EBA-175, which preferentially binds the Neu5Ac pathway (10). It is also likely that there would have been an intermediate stage, wherein EBA-175 of the *P. reichenowi* ancestor would have relaxed its specificity to accommodate binding of Neu5Ac. The final EBA-175 mutations potentially responsible for the malignancy and rapid expansion of *P. falciparum* may have occurred relatively recently, perhaps 5,000–10,000 years ago, which would account for the scarcity of neutral DNA polymorphisms in *P. falciparum*, consistent with a recent worldwide population expansion of this parasite (11, 12, 18, 22, 25).

Our investigations suggest that *P. falciparum* has only once established itself among human hosts. The zoonotic origin of *P. falciparum* elevates interest in the possible ongoing transmission of other malaria parasites of primate origin into the human population (37). The repeated emergence of human malaria parasites from zoonotic reservoirs raises the question of whether ongoing transmission of *P. reichenowi* from chimpanzees to humans may be possible (or vice versa). The fact that this transmission has not happened repeatedly may reflect the difficulty in changing the sialic acid binding specificity of the parasite-binding proteins. In this regard, it is interesting that a major barrier limiting cross-transmission of avian influenza into humans (and vice versa) is due to differences in sialic acid linkage binding specificity. In this case, it is the relative preference of the human and avian virus hemoglutinins for binding α-2–6- and α-2–3-linked sialic acids, respectively, on epithelial cells in target tissues (38–41). This is also another instance wherein a human-specific change in sialic acid biology is relevant to infectious disease transmission, as chimpanzees and other great apes do not express human upper airway epithelial α-2–6-linked sialic acid targets for human influenza viruses (42, 43).

Materials and Methods

Sample Collection and Preparation. In Côte d’Ivoire, tissue and blood samples were collected from ten chimpanzees that had died due to anthrax (44), respiratory disease (45), or other reasons in the research area of Tai National Park between 1998 and 2002 (46). DNA extractions from tissue were performed by using DNAeasy tissue kits (Qiagen). DNA was stored in several aliquots.

In Cameroon, samples were collected from captive animals in three wildlife sanctuaries. Animals were primarily wild-born and brought to the sanctuaries by authorities for identification by authorities or abandonment by owners. Blood samples were collected during routine health examinations, quarantine, or recaptures by sanctuary staff after escapes. Whole blood was collected via venipuncture into a syringe or EDTA vacutainer and 1 ml was spotted onto Whatman #3 filter paper or Whatman/S&S 903 filter paper. Spots were allowed to dry and were placed in an envelope and sealed in a cliplock bag with silica gel. Dried samples were frozen at −80 °C once in the laboratory in the US. Dry Blood Spot filter papers were processed with Epicenter Master Complete DNA and RNA purification kits (Epicenter Technologies) to obtain genomic DNA following the manufacturer protocols. Total DNA was first dissolved in 30 μL molecular grade H2O.

Gene Fragment Analysis. As noted by Martinsen et al. (47), reliable determination of species phylogeny is best accomplished by analyses of multiple gene fragments. Accordingly, we amplified gene fragments from the three principal genomes of Plasmodium parasites, mitochondrdial (cytB), apicoplast (clpC), and nuclear (18S rDNA). The 50-μL PCR used 1 μL stock DNA as a template, 1× PCR buffer containing 2.5 mM MgCl2, 0.2 mM dNTPs, 0.25 μM of each primer, and 1.25 unit TaqDNA polymerase. PCR for cytB gene used outer primers DW2 (TAATGCCATGACTTCTGATTTACG) and DW3 (TTCGCTGATCATACCATGAAAGATG), and inner primers DW1 (TCAAAGATGGTCCCTGTTG) and cytB1 (GACGATTCTGTGATCACGATATT), for amplification of genes encoding outer primers TFM1421++ (AAAAAAGTTAGGACAAAAAATTATA) and TFM1422++ (CAGACCTGATATAGGAAGT) (48), and inner primers CLPCF1 (TCTAAACATATTGTTGTCTCG) and CLPCRF1 (TTGGACAACTTAAATCTTGG). PCR for 18S
Testing Reciprocal Monophyly. Two means were used to evaluate whether different ancestors could likely have given rise to P. falciparum and P. reichenowii, based on the distribution of variation now evident in the cytB, clpC, and 185 genes. First, trees were searched under the criteria of maximum parsimony and maximum likelihood, with and without the imposition of a topological constraint consistent with the co-speciation hypothesis. The lengths and likelihoods of optimal trees identified in each case were compared. The cost of enforcing reciprocal monophyly, wherein the ancestor of P. reichenowii did not also give rise to P. falciparum, was judged to be considerable.

More formal statistical consideration of this cost was evaluated by means of Bayesian statistical sampling of the posterior distribution of trees identified under the null and alternative (co-speciation) hypotheses. For cytB, the HKY + G model was taken as a prior, which resembles the model identified as optimal by ModelTest (see above) but which also allows different rates of substitution for transitions and transversions. For the clpC and 185 genes, models identified by ModelTest (see above) were taken as priors. Tree topology and model parameters were allowed to vary in 10 million generations of an MCMC algorithm seeking optimal trees constrained to be consistent with the co-speciation hypothesis, or freed from any such constraint. After a “burn-in” period of 10 thousand generations assuming exponential demographic growth and a strict molecular clock, stable estimates of substitution parameters, tree topology, and tree likelihood were obtained. The ratio of the likelihood of each model, given the data, provided a means to evaluate the statistical cost of imposing either clade stability or intergrading over other plausible values for model parameters. The ‘weight of evidence’, representing the ratio of likelihoods, was represented by Bayes factors (28) calculated by using the methods implemented by BEAST v. 1.4.8 (27, 29).

ACKNOWLEDGMENTS. We thank the Cameroon Ministry of Forestry and Wildlife, Ministry of Science, Research and Innovation, Cameroon Wildlife Aid Fund, and Limbe Wildlife Centre for permits and permission to sample animals at Limbe Wildlife Centre, Mvog Beti Zoo, and Mfou National Park. We thank Dr. John Kiyang, Dr. Felix Lankster, Babila Talfon, Jean-Michel Takuo, Felix Nkom, Mary-Chantal Bindele, and Mamo Benadette Adzensus for Cameronal field support. We thank the Ministry of the Environment and Forests as well as the Ministry of Research, the directorship of the Tai National Park, and the Swiss Research Center in Abidjan in Côte d’Ivoire. For Ivoirian field support, we thank Ilka Hendrikx, F. Stumpf, Yasmin Mosteb, Cristina Gomes, Tobias Deschner, and Emmanuel Normand. Additional support was provided by the Robert Koch-Institut, the Max-Planck-Society, the Global Viral Forecasting Initiative, Google.org, and The Skoll Foundation. We are extremely grateful to Dr. François Renaud (Centre National de la Recherche Scientifique-International Relief and Development, Montpellier, France) for making available to us the DNA sequences of Plasmodium gaboni. Comments or assistance were provided by Norman Johnson, Dr. Mike Dean, Russell Hanson, Charles Wolfe, Dr. David Sintaatha, Lisa Krain, Bill Switzer, and Dr. Jared Diamond. We are grateful to Drs. François Renaud, Norbert Ansky, and Ajit Varki for helpful comments about the manuscript. Research and experimentation was funded by National Institutes of Health Grants NIH-RO1GM70077 and NIH-RO1GM60759 (to S.M.R.), with additional support from Cummings School of Veterinary Medicine at Tufts University. Support for collection of specimens in Cameroon was provided by the National Institutes of Health Director’s Pioneer Award DP1-OD00370 (to N.D.W.). The National Institutes of Health Fogarty International Center International Research Scientist Development Award (NIH-FIC D43TW00003-09) funded the National Geographic Society Committee for Research and Exploration.


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