

## Geographic Subdivision of the Range of the Malaria Parasite *Plasmodium vivax*

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We examined geographically distinct isolates of *Plasmodium vivax* and categorized them according to developmental success in *Anopheles albimanus*. We found that parasites from Central America and Colombia form a group distinct from those of Asia. New World isolates have a distinct chromosomal translocation and an episomal variation in the open reading frame (ORF) 470 DNA sequence that distinguishes them from the other isolates tested. Old World types of *P. vivax* were introduced into the Americas, and a remnant of this lineage remains in *P. simium*. It is indistinguishable from Old World *P. vivax* to the extent determinable by using our encoded markers and the examination of its developmental pattern in mosquitoes. The cohesive characteristics that separate types of *P. vivax* are predictors of range and potential for transmission and hence require taxonomic distinction.

*Plasmodium vivax* is the most common of four human malaria species, with a worldwide distribution within approximately 16° to 20° north and south of the summer isotherms. Before its unexplained disappearance from Europe, *P. vivax* was probably present as far north as Moscow. Currently the organism is endemic in many countries of Asia, the South Pacific, North Africa, the Middle East, and South and Central America (1).

The biologic diversity included within this species designation has justified the use of a trinomial system for naming, including the subspecies, a taxonomic character given formal recognition in the International Rules of Zoological Nomenclature. The concepts of “species” and “subspecies” are still hotly debated, camps often dividing between those referred to as lumpers and splitters. Although species has various definitions, most depend on whether populations share or do not share a common gene pool. A subspecies is a population or group of populations inhabiting a geographic subdivision of the range of a species and differing from other

populations by diagnostic morphologic characteristics. It follows that subspecies cannot be sympatric, as interbreeding would lead to the loss of identity (2). Some researchers think that distribution can be either geographically or ecologically based (3).

In this study, we describe a large number of New and Old World *P. vivax* isolates. To assess a measure of relationship between 10 different parasite isolates, we measured the parasite infectivity of >25,000 mosquitoes in a 10-year period, using a ratio of infectivity of *Anopheles albimanus* to that of an internal control as a measure of success. We also identified and monitored a genomic and an organellar polymorphic marker and applied these to 17 different isolates from a wide geographic range. Our results were totally consistent with the geographic separation based on developmental differences in the mosquito. We determined that independently isolated strains of New World *P. vivax* are very similar to each other and, as a group, are distinct from a broad distribution of isolates collected in Asia and Oceania. This is the first report of genomic, organellar, and phenotypic markers coalescing along geographic boundaries and justifies the naming of a new subspecies of *P. vivax*.

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## Geographic Differences in Development of *P. vivax* Isolates

We examined the transmissibility of different isolates of *P. vivax* and found that they vary in a selective way. The comparative feeding results from the study of 10 individual strains of *P. vivax* are shown (Figure 1). For all parasite strains collected from the New World infected mosquitoes (as measured by oocyst count), the average infection rate was 30.6% for a single line of Central American *An. albimanus* and 51.9% for a laboratory line of *An. freeborni*, the positive control. The Old World parasite strains, on the other hand, had an average infection rate of only 0.25% for *An. albimanus*, while infecting the positive control, *An. freeborni*, quite normally at 63.4%. Methods and preliminary data on the correlation between different mosquito colonies and parasite development in *An. culicifacies* have been reported (5).

Further experiments tested the effect of the origin of *An. albimanus* on transmissibility of *P. vivax*. Five New World *An. albimanus* colonies

were far more susceptible to each New World parasite than to any Old World parasite line (Table). The average infection rate for five different New World colonies was 21.2%. As the positive control, *An. freeborni* was infected by parasites from the different areas with a mean infection rate of 57.1%. Hence, the distinction of developmental success in *An. albimanus* did not relate to characteristics of a single colony but was a more general phenomenon. The single exception to the Old World-New World separation was *P. simium*, a New World monkey parasite morphologically similar to *P. vivax*, which has been reported to successfully infect *An. freeborni* but not to be very infective to *An. albimanus* (6).

## Separation of New and Old World *P. vivax* Malaria Indicated by Analysis of Nuclear-Encoded and Plastid DNA Markers

*P. vivax* isolates representing different geographic areas and one isolate of *P. simium*, a parasite of New World monkeys, were

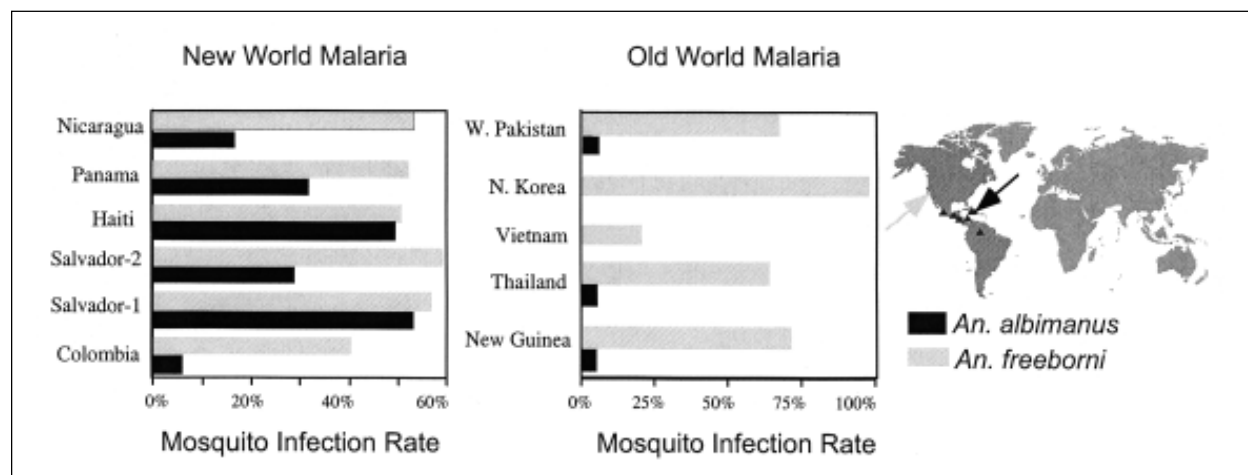


Figure 1. Comparison of the relative developmental success of 11 different isolates of *Plasmodium vivax* in *Anopheles albimanus* and *An. freeborni*. The black bars and arrow represent *An. albimanus*, and the gray bars and arrow represent *An. freeborni*. The origin of each isolate is indicated on the map. American Type Culture collection reference numbers are as follows: N. Korea T1206, Thai K1294, Vietnam C30151, New Guinea C30060, Nicaragua 30073, Panama C30138, and El Salvador-1 C30197. W. Pakistan, Salvador-2, and Colombia came directly from the Centers for Disease Control and Prevention (CDC). Analysis of feeding and infectivity are as described (4).\*

\*Susceptibility of *An. albimanus* to different strains of *P. vivax* was tested by feeding the mosquito on infected *Aotus* monkeys (4). A total of six strains of *An. albimanus* collected from Panama, El Salvador, Colombia, and Haiti were used to test each of 11 strains of *P. vivax*, 6 strains of which were from the New World, including 2 isolates from El Salvador and 1 each from Colombia, Haiti, Panama, and Nicaragua; 5 strains were from the Old World, including Chesson strain (New Guinea), West Pakistan strain (Pakistan), North Korea strain (Korea), Pakchong strain (Thailand), and Vietnam II strain (Vietnam). The infection rate was determined by dissecting and counting midgut oocysts or salivary glands during the second week after the infectious blood meal. *An. freeborni*, which were originally from Marysville, California, and have been maintained in the laboratory at CDC since 1944, were used as a positive control because this laboratory-selected colony has a high level of susceptibility to all strains of *P. vivax*.

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Table. *Anopheles albimanus* exhibits greater susceptibility to New World versus Old World *Plasmodium vivax*

<i>P. vivax</i>	Mosquito	Infection rate (No. positive/no. fed)
New World	<i>An. albimanus</i>	21.2% (5,888/27,700)
	<i>An. freeborni</i>	57.1% (13,227/25,555)
Old World	<i>An. albimanus</i>	0.4% (10/2,508)
	<i>An. freeborni</i>	57% (1,790/3,135)

characterized according to sequence polymorphisms within the nuclear-encoded rRNA genes and the 35-kb plastid genome. The New World *P. vivax* isolates were identical under these criteria, while the Old World *P. vivax* and *P. simium* also formed a distinct and related group. These parasites include most of the original parasite lines tested for developmental success in *An. albimanus*.

### Consistent Differences Between Old and New World Isolates of *P. vivax* in S-type rRNA Genes: Result of a Single-Type Gene Conversion

DNA sequence analysis of the cloned 18S rRNA genes from 17 isolates, including 5 of 7 Old World isolates and 5 of 7 New World isolates from infectivity studies, indicated that all strains shared similar sequence types, including 3 types of genomic rRNA genes as we described for the Sal-1 strain (7,8). In comparing the 18S rRNA sequences of New World and Old World isolates, however, we found a consistent polymorphism that separated the two according to geographic location (Figure 2). While the genes expressed in blood stage (A type) and oocysts (O type) remained the same in all isolates, the S-type genes transcribed in sporozoites were maintained in either one or the other of two distinct forms (Figure 2). The New World type of S gene has been reported in a *P. vivax* isolate from El Salvador (12), and the Old World type has been reported to exist in the monkey malaria, *P. simium* (13; Li and McCutchan, unpub. data). The difference between the two types of S gene is localized at the 3' end of the gene, including the last variable region, V8 (9). The A gene and two types of the S genes were differentiated on the basis of fragment length (Figure 2). The assignment of type was confirmed in all isolates by DNA sequence

analysis. The results indicate that one type of S gene is shared by all strains or isolates from Central and South America. Parasites from Asia, the Pacific region, and Africa have a genotype similar to the S gene from the primate malaria *P. cynomolgi* and exactly like that of *P. simium*.

The structure of the variant New World S gene appeared to be the result of a simple conversion between the A and S genes (Figure 3). Further, the point of conversion between A and S genes appeared to be the same (within 9 bp) in all New World isolates. The simplest conclusion is that only one conversion occurred, and it existed in the progenitor of all New World strains thus far examined. Expression of the two types of S gene has been confirmed by sequencing of cloned reverse transcriptase/polymerase chain reaction fragments amplified from sporozoite rRNA (Li and McCutchan, data not shown).

### Analysis of the Open Reading Frame (ORF) 470 in *P. vivax* Consistent with Parasite Associations Established with Genomic Markers

This 35-kb circular DNA is maternally inherited and highly conserved in sequence (14). We investigated the ORF 470, the small subunit rRNA, and the *Clp* gene for phylogenetically informative sites. The results show that a conserved substitution of the ORF 470 has been maintained among the New World *P. vivax*, where an isoleucine is replaced by valine. The parasites from Old World isolates maintain the isoleucine, as do their putative progenitors from primate malaras (Figure 4). The limited number of maternal types is not unexpected because of the extensive plastid homogeneity found within species of these parasites (14). We assume that this polymorphism is representative of two separate lineages.

### Discussion

The evolution of a new species or subspecies from within the range of a single sexually reproducing population has been of central interest to biologists since before Darwin. With regard to medicine and epidemiology, an understanding of the above would substantially contribute to designing procedures for pathogen control (15). We have shown that a partition subdivides the population range of *P. vivax* and have presented one reason why such partitions remain stably in place.

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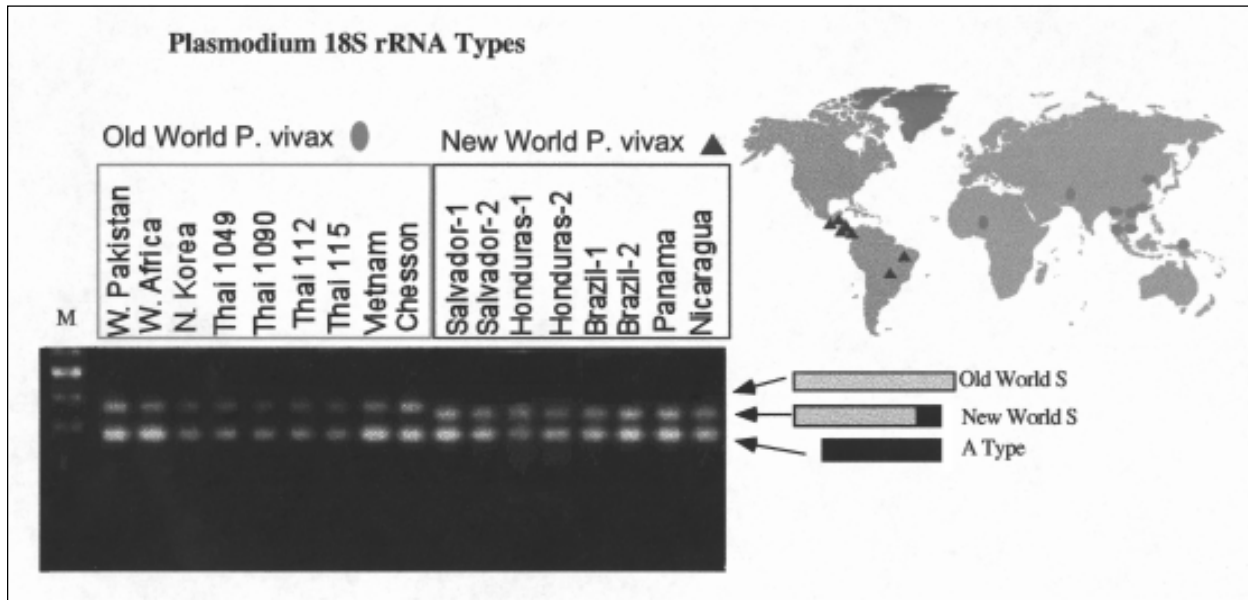


Figure 2. Sequences of *Plasmodium vivax* isolates are distinguished by variation in the 3' end of the S-type rRNA gene (9). The S-type gene is longer in Old World isolates and in *P. simium*. Oligonucleotide #902 (5'CAGCAAGCTGAATCGTAATTTTAA3') was used to detect type A rRNA, and #743 (5'ATCCAGATCCAATCCGACATA3') and #901 (5'GATAAGCACAAAATAGCGAAATGC3') were used to differentiate the two S-type rRNAs in membrane blot hybridization. American Type Culture Collection reference numbers not designated in the Figure 1 legend are as follows: Thai R112, Thai R115, Honduras-1 T09794, Honduras-2 T10595, Brazil-1 T40695, and Thai K1090. Haiti, Brazil-2, and the West African isolates came directly from the Centers for Disease Control and Prevention (CDC).\*†

\*The *P. vivax* SAL-1 strain, Africa strain, Pakistan strain, Brazil strain, and New World monkey vivax, *P. simium*, were from CDC. *P. vivax* Vietnam strain, Chesson strain, and Panama strain were from the American Type Culture Collection. The four Thai strains were from Walter Reed Medical Center; they were collected from four geographically separate locations in Thailand and their immunologic characteristics described with regard to the circumsporozoite protein gene (10). The heparinized blood samples were stored in 5% glycerolyte at -70°C.

†Purified DNA from frozen blood samples was processed with DNAzol reagents (GIBCO-Bethesda Research Laboratory, Gaithersburg, MD) according to the manufacturer's instructions. The partial sequence of 18S rRNA genes that covers variable regions 7 and 8 was amplified with a pair of genus-conserved primers, #841 and #844 (9). The sequence of an open reading frame (ORF 470) on the 35-kb plastid-like DNA was amplified with a pair of oligonucleotide primers #1274 (5' GTAAAAT TATATAAACCACC 3') and #1273 (5' GCACAATTTGAACGTAC 3') conserved in plastid-like organelle in *Apicomplexa* (11). The sequence of circumsporozoite protein genes was amplified with oligonucleotide primers #1157 (5' AATGGAGTAAACT TCAATAATGTA 3') and #1160 (5' CTCCACAGGTTACTACTGCATG 3'). The reaction was set up in a 100- $\mu$ L reaction volume containing 20–50 ng DNA, 200 M of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 2 mM MgCl<sub>2</sub>, and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) at a three-step cycling with the following parameters: 94°C/1 min for denaturation, 50°C/1 min for annealing, and 72°C/1–2 min for elongation for a total of 30 cycles. Conditions for separation of polymerase chain reaction products on agarose electrophoresis and <sup>32</sup>P-ending labeled probe hybridization were described (8,9). Oligonucleotide #902 (5' CAGCAAGCTGAATCGTAATTTTAA 3') was used to detect type-A rRNA, and #743 (5' ATCCAGATCCAATCCGACATA 3') and #901 (5' GATAAGCACAAAATAGCGAAATGC 3') were used to differentiate the two S-type rRNAs in membrane blot hybridization. Oligonucleotide #1163 (5' AGAGCAGCTGGACAGCCAGCA 3') and #1165 (5'GCWGGCAATCAACCAGGAGCA3') were used as probes to differentiate Sal-1 (classic) and PVK247 (variant) types of circumsporozoite protein genes, respectively. The gene coding for 18S ribosomal RNA was amplified from the DNA of Thai isolates with oligonucleotides #566, 5' GGATAACTACGGAAAAGCTGTAGC 3', and #570, 5' CGACTTCTCCTTCCTTTAAAA GATAGG 3', as the 5' and 3' end primers, respectively. Both primers are conserved for the genus *Plasmodium* and cover most of the transcribed sequences from approximately 140 downstream of the 5' end to 40 bp upstream of the 3' end.

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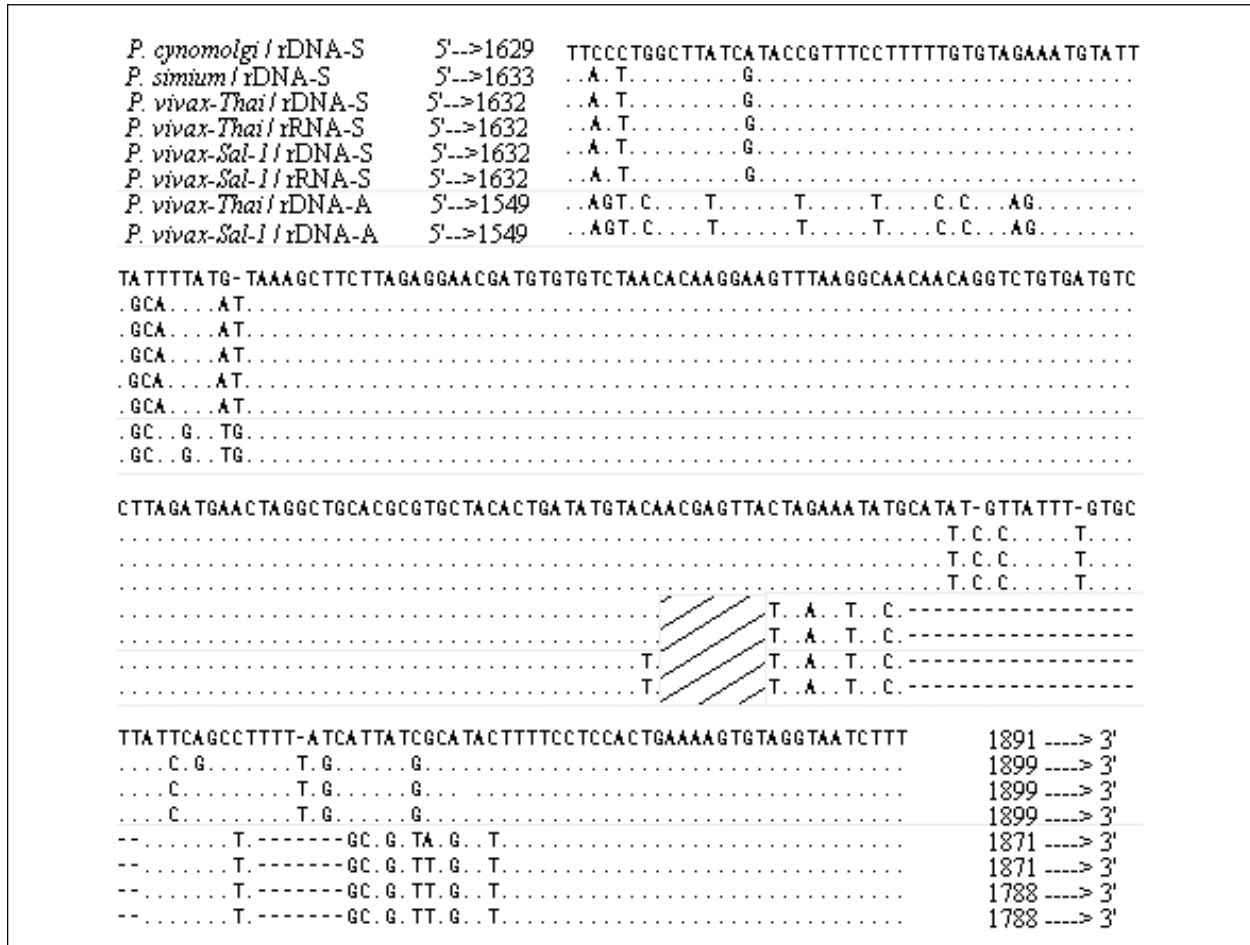


Figure 3. The sequence of *Plasmodium vivax* from the Americas is distinguished from Old World isolates by analysis of the 3' end of the S-type rRNA gene. The S-type rRNA sequences were determined from cloned amplified products of parasite DNA and RNA.

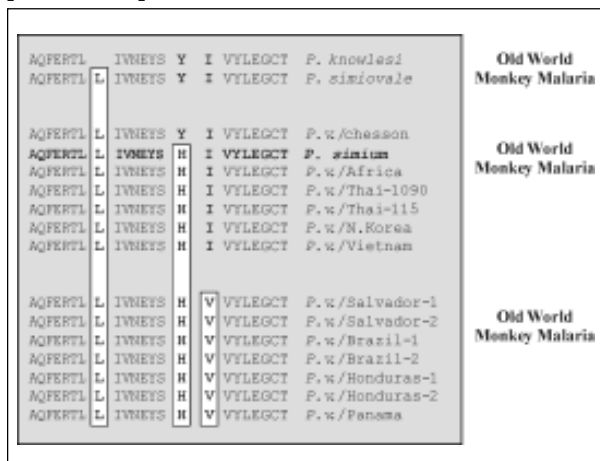


Figure 4. Polymorphism in the ORF 470 region of the 35-kb plastid-like DNA was determined by DNA sequence analysis after amplification of DNA from each isolate with oligonucleotide primers #1274 (5'GT AAAATTATATAAACACC3') and #1273 (5'GCACA ATTTGAACGTAC3') (11).

The epidemiologic impact of barriers to genetic exchange is substantial. Such barriers can come from both geologic and biologic sources. We have shown that differing vector-parasite compatibility can create genetic isolation between populations of a single parasite species. In principle, the question of differing vector-parasite compatibility has been addressed by the observation of historic events. When millions of potentially exposed soldiers returned home from World War II, Korea, and Vietnam, reintroduction of malaria was considered a serious possibility, given that *P. vivax* was only eliminated from the United States in the 1940s and the vectors were still in abundance. The fact that *P. vivax* was not reintroduced was, at the time, surprising. How could the parasites from Asia and America be different? Because *P. vivax* collected from the New World, Africa, and Asia have indistinguishable morphologic features and

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life cycles, as well as many of the same surface antigens, it was assumed that they represented the same parasite.

A dichotomy is revealed when one compares the relative developmental success of different parasite isolates in *An. albimanus* and *An. freeborni*. Both mosquitoes come from the Americas, but *An. albimanus* is indigenous to malarious areas and *An. freeborni* is not. When different isolates of *P. vivax* were fed to the New World mosquitoes, an obvious division formed along broad geographic lines. *An. albimanus* were more easily infected by New World parasites than by Old World parasites, whereas no difference in infectivity was detected in the control, *An. freeborni*. Hence, adaptation to the mosquito, rather than general developmental fitness, is the selective basis for separation. Examination of *An. albimanus* from five different areas of the New World each revealed the dichotomy. It is of interest that the insect-parasite association developed along broad geographic lines.

More than 25,000 mosquitoes were dissected during a 10-year period, and the resultant large numbers have implications for the interpretation of results. Clearly, the feedings were repeated many times, and each can be considered a separate experiment. The same protocol was followed in each experiment, but slight variations in factors such as the exact age of the mosquitoes and the source of infective blood must have occurred during the study period. The simple conclusion that a large difference exists in the developmental success of parasites from the Old and New Worlds in *An. albimanus* was entirely consistent over the period, and all experiments are in accord with that conclusion. Development of the same parasite strains was also tested in an Old World mosquito, *An. culicifacies* (Collins et al., unpub. data). As a group, Old World parasites infected *An. culicifacies* more efficiently and had a higher rate of oocyst production than parasites from the New World, although some New World parasite lines had not lost the potential to infect *An. culicifacies*. This indicates that while the lineage giving rise to New World parasites may have developed successfully in *An. culicifacies* and other Old World mosquitoes, that potential may be lost during the process of adapting to a new environment.

The developmental mechanism involved in determining success of a parasite isolate in mosquito colonies represents an unknown

number of loci. Data supporting the division defined by infectivity studies were based on DNA polymorphisms in samples supplied from the American Type Culture Collection (Rockville, MD) and the Centers for Disease Control and Prevention's Division of Parasitic Diseases. The episomal marker is located within the ORF 470 of the 35-kb plastid (11), which is physically unlinked to genomic markers. Our genomic marker is a polymorphism resulting from a defined ribosomal RNA gene transformation. Although we cannot definitively say that the division of groups is related to their genetic isolation (i.e., we cannot show that we have saturated the genome with markers), that does appear to be the case. The premise of our interpretation is that episomal and chromosomal ribosomal markers are not linked to each other and are unlikely to be linked to genes controlling parasite development in the mosquito.

*P. simium* carries genomic and maternal polymorphisms identical to the Old World human parasite *P. vivax*. Its ancestors are certainly more maternally related to the Old World *P. vivax* of humans than to the *P. vivax* that now dominates in humans in South America. Thus, a lateral transfer occurred during *P. simium* evolution, as previously indicated (13,16,17). *P. simium* and New World *P. vivax* did not originate directly from the same source, contrary to published speculation on the parasite's evolution. Therefore, *P. vivax* most likely entered the New World on two separate occasions and from different geographic locations.

Two types of repetitive epitope have been described in the circumsporozoite protein gene of *P. vivax*; their distribution appears to be worldwide (18). We confirm this finding in our isolates (data not shown); hence, all our isolates arose from a common ancestor. The separation of these two *P. vivax* subspecies may have started before entry into the Americas, but it is likely that isolation and adaptation to the new environment were strong forces in driving the fixation of the parasites that now exist there. It is widely, although not universally, agreed that *P. vivax* entered into the Americas within the last 500 years. If true, this would suggest that fixation and dispersal over a wide geographic area happened within a few hundred years. Although this seems rapid, it is within the realm of what we know about malaria. The period of isolation needed for subspecific differentiation

can occur in fewer than 300 generations. In a natural system involving *Plasmodium*, this could happen in as few as 60 years (19). Speculation with regard to the evolution of malaria parasites of neotropical monkeys suggests that altered selective pressures on an isolated group of mammalian malaria parasites may lead to the evolution of a new species in only a few hundred years. The time of genetic separation needed to form new species is thus generously provided within the above range.

*P. vivax* of the New World clearly has distinctive features that relate to its potential for spread and thus require taxonomic distinction. Justifications for the designation of subspecies are met;<sup>1</sup> phenotypic differences exist among parasites that occupy different sectors of the inclusive geographic range of *P. vivax*. There are subspecies of Old World *P. vivax* separated on the basis of biologic characteristics: relapse pattern (e.g., *P. vivax hibernans* and *P. vivax multinucleatum*) and morphologic characteristics such as multiple-cell invasion (20). It follows that if these were sympatric, interbreeding would have led to the loss of identity (2). The question is whether species classification is justified for the New World parasite. Historically, a major branching occurred in the *P. vivax* species, or species complex, which divided the New World *P. vivax* from known Old World parasites. The problem with either designation (species<sup>2</sup> or subspecies) is that it is unclear how many other taxonomic distinctions are warranted within the New World. For example, a study in Mexico links a polymorphism within the circumsporozoite protein gene with transmission and geographic distribution (21). Although this does not indicate the existence of other restricted gene pools, the circumsporozoite protein gene and transmission being genetically associated, it does show that further subdivisions are present within the New World that are being affected by mosquito distribution. We suggest that the New World parasite be given a subspecies designation, *P. vivax collinsi*, until it can be determined whether the New World group can be further

subdivided into phenotypically defined groups occupying portions of this parasite's range. The species designation *P. collinsi* should then be considered.

### Acknowledgments

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Dr. Li was a senior postdoctoral fellow in the Laboratory of Parasitic Diseases at the National Institutes of Health during the course of this work. He is now in his second-year residency in pathology at New York University. Upon completion of his residency, he plans to continue his research efforts in infectious diseases.

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<sup>1</sup>The biologic diversity inherent in *P. vivax* already justifies the use of a trinomial system for naming its members that includes the designation of subspecies, a taxonomic character given formal recognition in the International Rules of Zoological Nomenclature. A subspecies is a population or group of populations inhabiting a geographic subdivision of the range of a species and differing from other populations by diagnostic morphologic characteristics.

<sup>2</sup>The designation of separate species does not require that the two organisms cannot mate and produce viable progeny, only that this does not happen with frequency in natural situations.

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