Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people

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Malaria therapy, experimental, and epidemiological studies have shown that erythrocyte Duffy blood group-negative people, largely of African ancestry, are resistant to erythrocyte *Plasmodium vivax* infection. These findings established a paradigm that the Duffy antigen is required for *P. vivax* erythrocyte invasion. *P. vivax* is endemic in Madagascar, where admixture of Duffy-negative and Duffy-positive populations of diverse ethnic backgrounds has occurred over 2 millennia. There, we investigated susceptibility to *P. vivax* blood-stage infection and disease in association with Duffy blood group polymorphism. Duffy blood group genotyping identified 72% Duffy-negative individuals ($FY*B^{ES}/*B^{ES}$) in community surveys conducted at eight sentinel sites. Flow cytometry and adsorption–elution results confirmed the absence of Duffy antigen expression on Duffy-negative erythrocytes. *P. vivax* PCR positivity was observed in 8.8% (42/476) of asymptomatic Duffy-negative people. Clinical vivax malaria was identified in Duffy-negative subjects with nine *P. vivax* monoinfections and eight mixed *Plasmodium* species infections that included *P. vivax* (4.9 and 4.4% of 183 participants, respectively). Microscopy examination of blood smears confirmed blood-stage development of *P. vivax*, including gametocytes. Genotyping of polymorphic surface and microsatellite markers suggested that multiple *P. vivax* strains were infecting Duffy-negative people. In Madagascar, *P. vivax* has broken through its dependence on the Duffy antigen for establishing human blood-stage infection and disease. Further studies are necessary to identify the parasite and host molecules that enable this Duffy-independent *P. vivax* invasion of human erythrocytes.

erythrocyte | evolution | DARC | Madagascar

During malaria fever therapy trials, performed to treat neurosyphilis (1920s to 1960s) and in experimental field trials, it was consistently demonstrated that Africans and African-Americans were highly resistant to *Plasmodium vivax* blood-stage malaria when challenged with human blood or mosquitoes infected with limited numbers of *P. vivax* strains (1–3). Following identification of the Duffy blood group (Fy; reviewed in Zimmerman, 2004) (4), population studies showed that individuals of African ancestry expressed neither Fy^a nor Fy^b antigens and were classified as Duffy negative, Fy(a–b–) (5). Following on observations that vivax malaria was rare in Africa (6), Miller et al. performed definitive in vivo studies to show that Duffy-negative people resisted, whereas Duffy-positive people were susceptible, to experimental *P. vivax* blood-stage infection following exposure to infected mosquitoes (7). This seminal work, and related *Plasmodium knowlesi* in vitro studies (7–9), established the paradigm that malaria parasites invade erythrocytes through specific "receptor"-based interactions and that the Duffy blood group was the receptor for *P. vivax*.

Resolution of molecular genetic factors responsible for Duffy blood group phenotypes has since been achieved. Erythrocyte Duffy negativity is explained by a single-nucleotide polymorphism (SNP) in a GATA-1 transcription factor binding site of the gene promoter (–33T → C) that governs erythroid expression (10). Variant antigens Fy^a, Fy^b, and Fy^{bweak} are associated with SNPs in the gene's coding region (11–14). Parallel studies identifying the *P. vivax* Duffy binding protein (PvDBP) (15–17) have provided the opportunity to dissect further the molecular interactions between parasite and host originally predicted by Miller (18, 19).

With the availability of molecular diagnostics, observations of *P. vivax* PCR-positive, Duffy-negative individuals have been made (20–22). PCR-positive samples have been reported in Brazil where there is significant admixture of Duffy-negative and Duffy-positive individuals (20, 21). However, the key biological evidence showing erythrocytes infected by *P. vivax* has not been provided. Indeed, PCR could potentially detect *P. vivax* merozoites released into the bloodstream by infected hepatocytes that are susceptible to the mosquito-transmitted sporozoites. Positive PCR is therefore not synonymous with presence of intraerythrocytic parasites. A recent Kenya-based study reported *P. vivax*-positive *Anopheles* mosquitoes within Duffy-negative populations and low density, microscopically positive blood smears in Duffy-negative children, which could not be confirmed (22). To follow these observations, a survey of >2,500 blood samples from West and central Africans living in nine malaria-endemic countries was conducted to evaluate the prevalence of *Plasmodium falciparum*, *P. vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. Results found only one *P. vivax*-positive sample from a Duffy-positive individual and the authors concluded that low numbers of Duffy positives in Africa are sufficient to maintain *P. vivax* transmission (23). Thus, the question of *P. vivax* blood-stage infection of Duffy-negative individuals in areas where Duffy-positive and -negative populations are established remains open.

Madagascar, the world's fourth largest island 250 miles off the East African coast, has been peopled by successive Austronesian and African migrations over the past 2,300 years (24, 25) (*SI Appendix A*, *Figs. S1 and S2*). There, potential exists for significant admixture among Duffy blood group phenotypes [Fy(a+b+), Fy(a+b–), Fy(a–b+), Fy(a–b–), Fy(a+b^{weak}); nomenclature descri-

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bed in *SI Appendix B*, *Table S1*], providing Duffy-negative individuals consistent natural exposure to infection by *P. vivax*. In this island biogeographical context, where species are subjected to unique selective pressures, we investigated the relationship between Duffy blood group polymorphism and *P. vivax* blood-stage infection and clinical malaria.

Results

Duffy Genotyping and *Plasmodium* Species Diagnosis. Among 2,112 asymptomatic school children seen at eight sites (February–April 2007), 709 were randomly selected. Of these, 382 (53.8%) children were self-identified to be of Asian origin, and 327 indicated they were of African origin. Both Duffy genotyping and *Plasmodium* species diagnostic assays were performed successfully for 661 children (Table S2). Overall, 72.0% (476/661) were genotyped as Duffy negative ($FY*B^{ES}/*B^{ES}$) and 28% (185/661) were Duffy positive (17.7% $FY*A/*B^{ES}$, 4.7% $FY*B/*B^{ES}$, 3.8% $FY*A/*A$, 1.7% $FY*A/*B$, and 0.1% $FY*B/*B$). Prevalence of each *Plasmodium* species was 16.2% *P. falciparum*, 13.0% *P. vivax*, 3.6% *P. ovale*, and 1.8% *P. malariae*; 5.2% of participants were infected with multiple species. Among Duffy-negative individuals 42 (8.8%) were *P. vivax* PCR positive based on the small subunit (SSU) rDNA assay, 32 of which were characterized as *P. vivax* monoinfections. All 42 *P. vivax* infections were confirmed by additional *Plasmodium* species PCR assays based on cytochrome oxidase I (COI) and/or PvDBP (*SI Appendix C*). *P. vivax* infection and Duffy genotype distribution among the school-age children are summarized in Fig. 1. Results show that the highest number of Duffy-negative individuals PCR positive for *P. vivax* were observed at Tsiroanomandidy ($n = 30$) and Miandrivazo ($n = 9$), study sites with the highest frequencies of Duffy-positive study participants (47.2 and 31.8%, respectively).

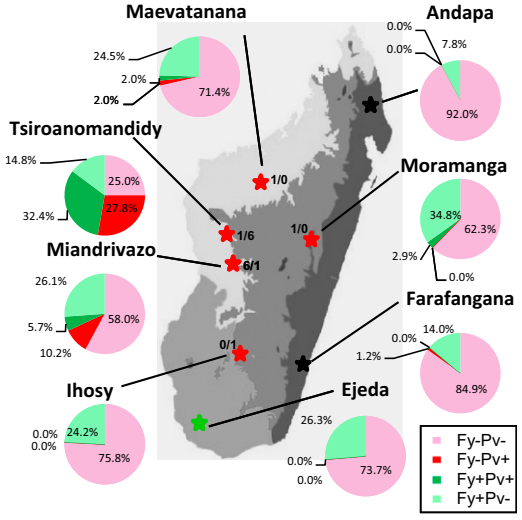

Fig. 1. Frequency distribution of *P. vivax* infections and clinical cases identified in Duffy-positive and -negative Malagasy people. Pie graphs show the prevalence of Duffy-positive (dark/light green) and Duffy-negative (red/pink quadrants) phenotypes in the eight Madagascar study sites. Prevalence of *P. vivax* infection observed in the survey of school-age children is shown in red and dark green; population subsets not infected with *P. vivax* are pink and light green. Study sites identified by a red star indicate that clinical vivax malaria was observed in Duffy-negative individuals (additional data classify clinical cases as numbers of people diagnosed with monoinfection *P. vivax*/mixed *P. vivax* + *P. falciparum*). A green star indicates that vivax malaria was observed in Duffy-positive individuals only (Ejeda). In Ihosy clinical malaria was observed in one individual with a mixed *P. vivax*/*P. falciparum* infection. *P. vivax* malaria was not observed in Andapa and Farafangana (black star). Malaria transmission strata are identified as tropical (lightest gray), subdesert (light gray), equatorial (middle gray), and highlands (dark gray).

Interestingly, in individual study sites with sufficient numbers of PCR-positive *P. vivax* infections to enable comparisons, prevalence ratios were not significantly different between Duffy-positive and -negative children (Tsiroanomandidy χ^2, 1 df = 2.87, $P = 0.09$; Miandrivazo χ^2, 1 df = 0.116, $P = 0.733$; Maevatanana χ^2, 1 df = 1.18, $P = 0.278$). In contrast to these village-specific findings, when considering all 661 school children surveyed, Duffy-negatives were still 3-fold less likely to experience a *P. vivax* blood stage infection compared to Duffy-positive children (Odds ratio = 0.310, 95% confidence interval 0.195–0.493; $P < 0.001$).

Intraerythrocytic *P. vivax* Infection and Clinical Malaria in Duffy Negatives. One hundred eighty-three *P. vivax*-infected samples were collected in 2006/2007 from febrile individuals seeking malaria treatment from health facilities (26). Among these, 153 carried *P. vivax* monoinfections and 30 were *P. vivax*/*P. falciparum* mixed infections. Of the patients experiencing clinical malaria, 17 Duffy-negative individuals from five of the study sites (Fig. 1, red stars; Table S3) were *P. vivax* infected. Whereas 8 of these malaria patients were infected with *P. vivax* in the context of a mixture of *Plasmodium* species, 9 were judged to be infected with only *P. vivax* by combined conventional blood smear and PCR-based *Plasmodium* species diagnoses. With 5.9% (9/153) of clinical *P. vivax* monoinfection malaria experienced by Duffy negatives vs. 94.1% (144/153) by Duffy positives, Duffy negativity conferred a >15-fold reduction in prevalence to *P. vivax* malaria.

Standard blood smear microscopy showed evidence of intraerythrocytic infection in four individuals by examining Giemsa-stained slides (Fig. 2). Classic morphological features of *P. vivax* trophozoites (Fig. 2 *A* and *B*) and *P. vivax* gametocytes (Fig. 2 *C–F*) were observed. Results showing both male and female gametocytes within the same infection suggest that *P. vivax* transmission from Duffy-negative people is possible.

Erythrocyte Duffy-Negative Genotype and Phenotype Concordance. Further evaluation of 43 Tsiroanomandidy school children was performed to confirm concordance between Duffy genotypes and phenotypes using conventional serology, flow cytometry, and adsorption–elution methods. Comparison of Duffy genotyping with serology was 100% concordant for all Duffy positive/negative and Fy^a/Fy^b antigenic classifications. Fig. 3 illustrates flow cytometry phenotypes comparing control donors and field samples. Fig. 3*A* shows mean fluorescence intensities (MFI) that reflect binding of the Duffy antigen-specific anti-Fy6 antibody (NaM185-2C3) for well-characterized control donors who were $FY*B^{ES}/*B^{ES}$ [Fy(a−b−)], $FY*B^{ES}/*X$ [Fy(a−b^{weak})], $FY*X/*X$ [Fy(a−b^{weak})], $FY*A/*B^{ES}$ [Fy(a+b−)], and $FY*A/*B$ [Fy(a+b+)], respectively. Additionally, Fig. 3*A* shows that flow cytometry results for one $FY*B^{ES}/*B^{ES}$ Malagasy donor were identical to the West African $FY*B^{ES}/*B^{ES}$ [Fy(a−b−)] control and the isotype background control, confirming no erythrocyte surface expression of the extracellular amino terminus of the protein known to mediate invasion of vivax merozoites. Fig. 3*B* shows results for 40 Malagasy individuals, with 30 samples genotyped $FY*B^{ES}/*B^{ES}$ [Fy(a−b−)], 6 genotyped $FY*A/*B^{ES}$ [Fy(a+b−)], 2 genotyped $FY*A/*A$ [Fy(a+b−)], and 2 genotyped $FY*A/*B$ [Fy(a+b+)]. Results show that Duffy antigen expression was uniformly absent from erythrocyte surfaces of all $FY*B^{ES}/*B^{ES}$ individuals; flow cytometry phenotypes for the Duffy-positive donors showed expected patterns of anti-Fy6 antibody binding. To ascertain that ablated serological detection of Duffy was not due to a mutation in the epitope-coding sequence, >2,550 bp of the Duffy gene were sequenced for 14 Duffy-negative Malagasy study participants who had experienced *P. vivax* clinical malaria (included proximal promoter and full coding sequence; GenBank accession nos. GU130196 and GU130197). This sequencing showed identity between Duffy-negative Malagasy alleles and three West African $FY*B^{ES}$ alleles and the $FY*B^{ES}$ GenBank

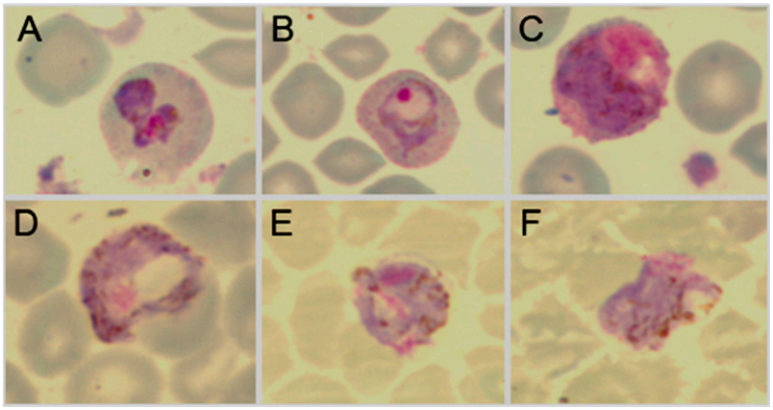

Fig. 2. Standard Giemsa-stained thin smear preparations of *P. vivax* infection and development in human Duffy-negative erythrocytes. *A–C* originated from a 4-year-old female, genotyped as Duffy negative ($FY{*}B^{ES}/{*}B^{ES}$), who presented at the Tsiroanomandidy health center (June 26, 2006) with fever (37.8 °C), headache, and sweating without previous antimalarial treatment. Standard blood smear diagnosis revealed a mixed infection with *P. vivax* [parasitemia = 3,040 parasitized red blood cells (pRBC)/μL] and *P. falciparum* (parasitemia = 980 pRBC/μL). PCR-based *Plasmodium* species diagnosis confirmed the blood smear result; *P. malariae* and *P. ovale* were not detected. *A* shows an undifferentiated *P. vivax* trophozoite with enlarged erythrocyte volume, clear evidence of Schüffner stippling, and amoeboid morphology. *B* shows a *P. vivax* early stage trophozoite with condensed chromatin, enlarged erythrocyte volume, Schüffner stippling, and irregular ring-shaped cytoplasm. *C* shows a *P. vivax* gametocyte: Lavender parasite, larger pink chromatin mass, and brown pigment scattered throughout the cytoplasm are characteristics of microgametocytes (male). *D* originated from a 12-year-old Duffy-negative ($FY{*}B^{ES}/{*}B^{ES}$) male, who presented at the Miandrivazo health center (June 27, 2006) with fever (37.5 °C) and shivering without previous antimalarial treatment. Standard blood smear diagnosis and light microscopy revealed infection with only *P. vivax* (parasitemia = 3,000 pRBC/μL). PCR-based *Plasmodium* species diagnosis confirmed this blood smear result; *P. falciparum*, *P. malariae*, and *P. ovale* were not detected. The parasite featured shows evidence of a *P. vivax* gametocyte: Large blue parasite, smaller pink chromatin mass, and brown pigment scattered throughout the cytoplasm are characteristics of macrogametocytes (female). *E* and *F* originated from a 3-year-old Duffy-negative ($FY{*}B^{ES}/{*}B^{ES}$) female, who presented at the Moramanga health center (April 11, 2006) with fever (37.8 °C) without previous antimalarial treatment. Standard blood smear diagnosis and light microscopy revealed infection with only *P. vivax* (parasitemia = 3,368 pRBC/μL). PCR-based *Plasmodium* species diagnosis confirmed this blood smear result; *P. falciparum*, *P. malariae*, and *P. ovale* were not detected. The parasites featured show additional evidence of *P. vivax* gametocytes.

reference sequence (X85785) (10) and verified that failure to detect the Duffy antigen by serology resulted from the −33 T → C GATA-1 promoter mutation of the otherwise unaltered Duffy gene.

***P. vivax* Strains Infecting Duffy-Negative Malagasies.** To evaluate the diversity of *P. vivax* strains we analyzed the circumsporozoite protein (PvCSP) and *P. vivax*-specific microsatellites (27). Positive genotyping of 16 isolates for PvCSP showed the presence of both VK210 and VK247 variants (VK210, $n = 6$; VK247, $n = 1$; VK210 and VK247, $n = 9$). The mean Nei's unbiased expected heterozygosity (H_e) estimated with microsatellite loci (6–13 alleles identified per infection) did not differ significantly between Duffy-positive ($n = 45$) and Duffy-negative ($n = 11$) patients (0.67 ± 0.17 vs. 0.74 ± 0.15, $n = 11$, $P > 0.05$); Wright's fixation index analysis showed an absence of genetic differentiation between the two populations ($F_{st} = 0.0094$, $P = 0.20$). Results suggest that numerous strains are able to infect both Duffy-negative and -positive individuals.

Discussion

We studied *P. vivax* infection in Madagascar in an admixed human population that included Duffy-positive and -negative people to test whether consistent natural exposure may have provided *P. vivax* with sufficient opportunity to break through the Duffy-negative barrier thought to confer human resistance to *P. vivax* blood-stage infection. We observed eight different Duffy genotypes in the study population, including a high frequency of Duffy negativity (72%), and discovered that a considerable number of Duffy-negative Malagasies were susceptible to *P. vivax* blood-stage infection and clinical vivax malaria. Vivax malaria was most common in study sites where the prevalence of Duffy positivity was highest. Whereas we found that *P. vivax* PCR positivity did not differ between Duffy-positive and -negative people in high prevalence villages (Tsiroanomandidy and Miandrivazo), we observed a significant 3-fold reduction in *P. vivax* infection in Madagascar overall, and a substantial reduction in prevalence of clinical *P. vivax* malaria among Duffy negatives compared to Duffy positives. These findings suggest that *P. vivax* invasion of Duffy-negative erythrocytes may be somewhat impaired relative to invasion of Duffy-positive individuals, preventing development of higher parasitemia associated with clinical disease in many individuals.

Human settlement of Madagascar from populations participating in the Indian Ocean trade network beginning ≈2,000 years ago may have been responsible for introducing *P. vivax* into Madagascar by infected immigrants from Southeast Asia (*SI Appendix A*). Increase in human population density would have provided conditions to sustain *P. vivax* transmission. It is unlikely that *P. vivax* would cause blood-stage infection in Duffy negatives initially. However, a consistent supply of parasites available from infected Duffy-positive Malayo-Indonesians would have provided ample opportunity for infection of hepatocytes of Duffy negatives and selection of *P. vivax* strains with a new capacity for erythrocyte invasion. A report that the so-called Madagascar *P. vivax* strain (28) caused blood-stage infection in one Liberian individual (2) may provide evidence of unique *P. vivax* evolution in Madagascar consistent with our findings, although the Duffy phenotype of the susceptible Liberian was not established.

Whereas new combinations of mutations or altered gene expression could have resulted from population admixture and subsequent recombination between Duffy-negative and -positive alleles in the study participants, Duffy gene sequence analysis and flow cytometry results provide no evidence that a Duffy receptor is available on erythrocyte surfaces of genotypically Duffy-negative ($FY{*}B^{ES}/{*}B^{ES}$) Malagasies. Thus *P. vivax* strains infecting Duffy negatives in this study would have required a Duffy-independent mechanism for erythrocyte invasion.

Interestingly, observations of *P. vivax* infection of nonhuman primate erythrocytes and human infection by the related *P. knowlesi* may provide insight regarding an alternative invasion mechanism.

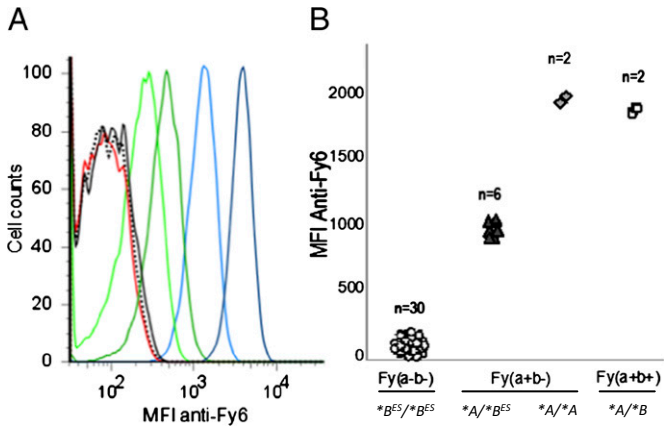

Fig. 3. Flow cytometry and serological correlation of Duffy-negative and Duffy-positive phenotypes with respective genotypes in Malagasy school children from Tsiroanomandidy, Madagascar. (*A*) Flow cytometric analysis of Duffy blood group genotypes. Flow cytometry histograms show MFI that reflect binding of the Duffy antigen-specific anti-Fy6 antibody (NaM185-2C3) for one Malagasy genotyped as *FY*B^ES/*B^ES* [Fy(a−b−)] (red) and five well-characterized control donors who are *FY*B^ES/*B^ES* [Fy(a−b−)] (solid black), *FY*B^ES/*X* [Fy(a−b^{weak})] (light green), *FY*X/*X* [Fy(a−b^{weak})] (green), *FY*A/*B^ES* [Fy(a+b−)] (light blue), and *FY*A/*B* [Fy(a+b+)] (blue), respectively. Results include fluorescence of a Duffy-positive blood sample incubated with an isotype control antibody (dotted black line). (*B*) Flow cytometry of Duffy antigen expression on erythrocytes from 40 Malagasy study participants. Flow cytometry results show MFI that reflect binding of the Duffy antigen-specific anti-Fy6 antibody for 30 *FY*B^ES/*B^ES* [Fy(a−b−)] Malagasy samples (mean = 48, SD = 1.2), 6 *FY*A/*B^ES* [Fy(a+b−)] Malagasy samples (mean = 1,025, SD = 22.4), 2 *FY*A/*A* [Fy(a+b−)] Malagasy samples (mean = 1,937, SD = 54) and 2 *FY*A/*B* [Fy(a+b+)] Malagasy samples (mean = 1,896, SD = 8).

P. vivax readily infects erythrocytes of the squirrel monkey (*Saimiri boliviensis*) (29, 30). Whereas squirrel monkeys express an Fy6-positive Duffy antigen (29–31), the *P. vivax* DBP binds poorly, if at all, to squirrel monkey erythrocytes (32), suggesting a PvDBP-independent invasion mechanism. In vitro studies showing that *P. knowlesi* invades Duffy-negative erythrocytes treated with trypsin and neuraminidase (33) suggest that *P. knowlesi* possess additional erythrocyte invasion ligands enabling Duffy-independent blood-stage infection. Whether our results signal local evolution of a new *P. vivax* erythrocyte invasion pathway, or indicate the existence of yet-uncharacterized erythrocyte invasion mechanisms involving DBPs and/or reticulocyte binding proteins (34, 35), remains to be clarified.

With accumulating reports on severe *P. vivax* morbidity and mortality there is a growing appreciation that this parasite exerts considerable selective pressure on human health (36). Meanwhile, debate persists regarding the evolutionary relationship between *P. vivax* and Duffy negativity. Observations of *P. vivax* PCR positivity in Duffy-negative people add support for alternative receptors (20–22). In contrast, the observation that carriers of the Papua New Guinea Duffy-negative allele (*FY*A^ES*) (37) experience reduced *P. vivax* blood-stage infection (38) underscores the strong dependence this parasite displays on Duffy-dependent invasion.

Our observations in Madagascar showing conclusive evidence that *P. vivax* is capable of causing blood-stage infection and disease in Duffy-negative people illustrate that in some conditions *P. vivax* exhibits a capacity for infecting human erythrocytes without the Duffy antigen. The data assembled in this study suggest that conditions needed to clear the barrier of Duffy negativity may include an optimal human admixture. In Madagascar with significant numbers of Duffy-positive people and full susceptibility of hepatocytes in Duffy negatives, *P. vivax* may have sufficient exposure to Duffy-negative erythrocytes, allowing more opportunities for de novo selection or optimization of an otherwise cryptic invasion pathway that nevertheless seems less efficient than the Duffy-dependent pathway.

Finally, given our observations in Madagascar and those from South America and Kenya, a better understanding of the alternative pathways *P. vivax* uses to invade human erythrocytes should become a priority. As current *P. vivax* vaccine strategies focused on PvDBP attempt to exploit Duffy-dependent invasion (39), these collected findings emphasize the importance of a multivalent vaccine strategy that can reduce the potential for parasite strains to escape immunologic control focused on a single protein and a single erythrocyte invasion pathway.

Materials and Methods

Populations and Conventional Parasite Diagnosis. Human subjects protocols (007/SANPF/2007 and 156/SANPFPS/2007) were approved by the Madagascar Ministry of Health, National Ethics Committee; genotyping was performed following a University Hospitals Case Medical Center Institutional Review Board protocol (08-03-33). A cross-sectional survey to evaluate erythrocyte polymorphisms associated with malaria susceptibility was conducted among Malagasy school children in 2007 (40). Children (3–13 years) were recruited at eight study sites, representing the four malaria epidemiological strata of Madagascar (*SI Appendix A*), using a two-level cluster random sampling method (school and classroom). After obtaining informed consent from parents/guardians, whole blood (5 mL) was collected (K^+-EDTA Vacutainers) by venipuncture from each child. In March 2009 additional blood samples were collected from the same Tsiroanomandidy study population.

In vivo efficacy studies on antimalarial drugs were conducted in 2006 and 2007 at the eight study sites (registration no. ISRCTN36517335) (26, 27). *P. vivax* clinical samples, collected on filter paper, were selected from all patients screened by a rapid diagnostic test (RDT) (OptiMAL-IT; Diamed AG). Giemsa-stained thin/thick blood films were prepared for each RDT-positive patient to check both *Plasmodium* species identification and parasite densities. All patients enrolled in these studies were >6 months old, judged to be *P. vivax* positive with parasite densities ≥250/μL, and had a history of fever (axillary temperature ≥37.5 °C) 48 h before recruitment. Patients displaying mixed infections with *P. vivax* and *P. falciparum* were treated according to the new National Malaria Policy, with a combination of artesunate and amodiaquine (Arsucam) (41). An enrollment questionnaire administered to each patient included history of fever, prior treatment, age, gender, location of habitation, and ethnicity.

DNA Extraction. DNA was extracted from blood spots with Instagene Matrix resin (BioRad) or directly from whole blood (100 μL) using proteinase K/phenol-chloroform.

Molecular Diagnosis. Molecular diagnosis evaluating SNP (Duffy −33, promoter ±; codon 42, *FY*A* vs. *FY*B*) was performed using a post-PCR ligase detection reaction–fluorescent microsphere assay (LDR-FMA) or direct sequencing of PCR products (*SI Appendix C*).

Plasmodium species identification from school children was performed using a PCR-based SSU rRNA assay (42). Asymptomatic *P. vivax* infections were confirmed for each Duffy-negative sample using PCR-based assays for COI and PvDBP. *P. vivax* population diversity was evaluated using PvCSP and microsatellite markers (27). *Plasmodium* species identification from clinical samples was performed using real-time (43) and classical PCR (44).

Duffy Phenotyping. Duffy phenotyping was performed using fresh blood samples collected in March 2009. Duffy antigens (Fy^a/Fy^b) were phenotyped using a microtyping kit and antisera (DiaMed-ID Microtyping System), following manufacturer's instructions. Expression of Duffy antigen on erythrocytes was evaluated by flow cytometry (BD FACS Canto II flow cytometer; Becton Dickinson) using monoclonal antibodies: F655 antibody (Fy^a specific), Hiro31 antibody (Fy^b specific), and anti-Fy6 antibody (NaM185-2C3 clone, Duffy specific) (45). Briefly, erythrocytes from EDTA-anticoagulated field and control samples [Fy(a+b−), Fy(a−b+), Fy(a+b+), and Fy(a−b^{weak}); obtained from the Centre National de Reference pour les Groupes Sanguins, Paris] were washed twice in phosphate buffer solution (PBS). Cells were then resuspended with isotype controls IgG/IgM (5 μg/mL; BD) or monoclonal antibodies (anti-Fy6 diluted at 1:8, Hiro31 and F655 diluted at 1:4) at room temperature for 1 h in PBS/0.1% BSA solution. After primary incubation, cells were washed twice in PBS and incubated in the dark at room temperature for 1 h with secondary phycoerythrin (PE) antibody (Beckman Coulter) at a concentration of 5 μg/mL in PBS/0.1% BSA solution. After a final wash in PBS, cells were

acquired by a digital high speed analytical flow cytometer. Erythrocytes were identified on the basis of forward/side scatter characteristics, using logarithmic amplification. After excitation at 488 nm, PE signal was collected with a 585/42 band pass filter. Data were acquired by BD FACS Diva software (v6.1.2) and analyzed using FlowJo (Treestar) software v7.2.5. Final controls of erythrocyte Duffy antigen expression were performed using adsorption–elution experiments (14).

Population Genetic Analyses. Genetic diversity was assessed by Nei's unbiased expected heterozygosity (H_e) from haploid data and calculated as $H_e = [n/(n-1)][1 - \sum p_i^2]$ (n is the number of isolates sampled; p_i^2 is the frequency of the ith allele) (46). Population genetic differentiation between symptomatic Duffy negatives and positives was measured using Wright's F statistics (47); population genetic parameters were computed with FSTAT software, v2.9.4 (48).

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Supporting Information

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SI Appendix A: Malagasy Study Sites, Peopling History, and Ethnicities

Location and Description of the Collection Sites, Madagascar, 2006–2007. The island nation of Madagascar (581,540 km^2) is located ≈400 km (250 miles) off the eastern coast of Africa across the Mozambique Channel (latitude 20°00′S, longitude 47°00′E). Madagascar's tropical climate is generally partitioned into rainy (December–April; rainfall 30–355 cm) and dry (May–November; average midday temperatures range from 25 °C in the highlands to 30 °C on the coast) seasons. Madagascar's population is an estimated 20 million (2008). Eight sites included in this study and partitioned into the four malaria epidemiological strata are identified in Fig. S1 (1).

Details of Individual Study Sites. ***Andapa—latitude 14°39′S, longitude 49°39′E.*** Andapa is characterized by an equatorial climate (average temperature, 22.5 °C; annual rainfall, 1,800 mm) with a stable transmission of malaria (entomological infection rate, EIR = 9) throughout the year. The majority of the population (20,000 inhabitants) is composed of the Tsimihety ethnic group with a minority of Antemoro, Betsimisaraka, Merina, and Betsileo.

Ejeda—latitude 24°21′S, longitude 44°31′E. Ejeda is characterized by a subarid climate (average temperature, 24.6 °C; annual rainfall, 580 mm). The malaria transmission is low (EIR = 0.2) and occurs during the rainy season (January–April). The majority of the population (26,000 inhabitants) is composed of the Mahafaly ethnic group.

Farafangana—latitude 22°49′S, longitude 47°50′E. Farafangana is characterized by an equatorial climate (average temperature, 26.8 °C; annual rainfall, 2,000 mm with 175 days of rain per year). Stable and high (EIR = 35–50) malaria transmission occurs throughout the year. The population (26,000 inhabitants) is composed of Antaisaka, Antaifasy, and Zafisoro ethnic groups.

Ihosy—latitude 22°24′S, longitude 46°08′E. Ihosy, located in the south of the Central Highlands, is characterized by grassy savannah plains (average temperature, 20 °C; annual rainfall, 700–900 mm). Malaria transmission is comparable to that observed in Ejeda. The population (17,000 inhabitants) is composed of Bara, Betsileo, and Antaisaka ethnic groups.

Maevatanana—latitude 16°56′S, longitude 46°49′E. Maevatanana is characterized by a tropical climate with a 6-month (November–April) hot/rainy season (average temperature, 27 °C; annual rainfall, 1,800 mm) and a 6-month dry season. The malaria transmission is intermediate compared to other sites (EIR = 3–10). The population (16,000 inhabitants) is composed primarily of Merina and Sakalava ethnic groups.

Miandrivazo—latitude 19°31′S, longitude 45°29′E. Miandrivazo, situated near to the river Mahajilo, is declared the hottest city in Madagascar with an annual average temperature of 28 °C. The malaria transmission is high (EIR = 32). The population is (20,000 inhabitants) composed of a majority of the Sakalava ethnic group and followed by Antaisaka and Betsileo ethnicities.

Moramanga—latitude 18°56′S, longitude 48°12′E. Moramanga is located in the eastern foothills of the central highlands (900-m altitude). Moramanga is characterized by austral climate (average temperature, 19.4 °C; annual rainfall, 1,500 mm). The malaria transmission is unstable and low (EIR = 2), with peak transmission from March to May. The population (26,000 inhabitants) is composed of the Bezanozano and Merina ethnic groups.

Tsiroanomandidy—latitude 18°77′S, longitude 46°04′E. Tsiroanomandidy is located in the northwest of the central highlands (900-m altitude). The average annual temperature is 22.5 °C and rainfall is ~1,616 mm. This is an important agriculture area and experiences high migration from surrounding locations seeking employment. The malaria transmission is moderate (EIR = 2.5) with a peak of transmission from March to May. The population (25,000 inhabitants) is composed primarily of the Merina and Betsileo ethnic groups.

Early History of the Malagasy People and Ethnicities. Peopling of Madagascar is recent in human history. Whereas much remains uncertain, the island of Madagascar has been settled by a wide range of ethnicities from diverse backgrounds to create a multicultural society including Southeast Asian (Indonesia), African, Middle Eastern, Indian, and European origins (2).

Human settlement of Madagascar (Fig. S2) is suggested to have been initiated by sea-faring people of Indonesia or Malaysia (Nias Island of western Sumatra or Borneo, respectively) with evidence that founding individuals arrived 2,300 years before present (YBP) (3). The earliest human activities in Madagascar have been localized to the southwest region near modern-day Toliary and the northeast region near Antsiranana. The earliest human travelers left traces of activity in these sites, suggesting brief visits but not colonization (4). Although difficult to substantiate, some authors believe that early migrants passed through India, South Arabia, and the East African coast. Upon Bantu migration (Tanzania and Mozambique) from Africa during the second and third centuries and new waves of Malayo-Indonesian immigration from the eighth century on, significant cultural assimilation and genetic admixture has occurred. The oldest known center of human colonization was in the northwest around the Islamic port of Mahilaka (present-day Amganja, Bay of Ampasindava), prosperous in the Indian Ocean trade network in the 12th–14th centuries (4). In this same time frame human occupation can be substantiated along the entire Madagascar coastline. Settlement of the central highlands areas was underway by the 10th century, but is suggested to have occurred with early Bantu and Indonesian settlers attempting to avoid unhealthy conditions along the coast including plague, malaria, and dysentery (3). The first European immigrants (Portuguese, Dutch, French, and English) began to appear starting in the 15th century. The colonial period from the late second millennium brought people from India and Asia to Madagascar to further strengthen the cultural miscegenation in this country, a true "melting pot" of the three continents of the ancient world (3).

Malaria is likely to have been transported to Madagascar through the earliest human settlers >2,000 years ago. It is more difficult to predict when during the first millennium of human settlement the human population numbers and density became favorable to support consistent transmission of the four common species of human malaria parasites that are observed in Madagascar today.

The relative contributions from the different ancestral founders to present-day culture and genetic polymorphism in Malagasy people have been the subject of debate among anthropologists and human geneticists (5, 6). Linguistic studies suggest that the Malagasy is most closely related to the Maanyan language from the Barito River region of central Borneo (7). Until the current study, human genetic studies have included mitochondrial DNA (mtDNA) (8, 9), Y chromosome (8) markers to assess maternal and paternal lineages, respectively, and sickle cell (β-globin; *HBB*) HbA and HbS polymorphisms (10) to study African and Asian haplotypes associated with exposure to malaria.

An initial study by Soodyall et al. focused on the 9-bp deletion found in mtDNA, originally found in Asian and New World populations, but later shown to have arisen independently in African populations; single-nucleotide polymorphisms in the mtDNA control region distinguish a Polynesian from the African motif associated with the deletion (9). Results from this study found that the

9-bp deletion was present in 26.8% of Malagasy people, 70.7% Polynesian/Asian derived and 29.3% African derived. Whereas the Polynesian motif was found in 18.2% of the Malagasy people surveyed, this sequence is not observed in Barito River populations. In more recent studies by Hurles et al. four different Malagasy ethnic groups (Bezanozano, $n = 6$; Betsileo, $n = 18$; Merina, $n = 10$; Sihanaka, $n = 3$; total Malagasy, $n = 37$) were compared to 327 samples representing major Southeast Asian population groups and 72 samples representing East African populations based on more extensive mtDNA and Y chromosome markers (8). Phylogeny of mtDNA variation partitions generally along lines observed almost exclusively in Africans (L lineages) and outside of Africa (M and N lineages). Results found that 23 (62.2) of the Malagasy samples were characterized by M or N lineages and 14 (37.8) by L lineages. The Y chromosomal lineages partition with no overlap between East Africa and Island Southeast Asia, and results from these studies suggested that 51% of the Y chromosomal lineages have an African origin. Whereas these results suggest a predominance of African paternal and Malaysian maternal ancestry, overall statistical analyses from these studies are inconclusive. F_{st} analysis using Arlequin software suggested that among the Malagasy samples exhibiting Asian Y chromosome haplotypes, two populations from Borneo are the best candidates for their likely origin. Interestingly, Asian influence is greater among Betsileo and Merinas, wheras it is lower in coastal populations.

Hewitt et al. focused their analyses on different haplotypes associated with b-globin polymorphisms, hemoglobin S (HbS), and wild-type hemoglobin A (HbA) alleles (study population $n = 1,425$) (10). Genetic evidence suggests that the HbS polymorphism has occurred independently five times through observation of associated Senegal, Benin, Bantu, Cameroon, and Arab-Indian haplotypes. In the survey by Hewitt et al., the Bantu haplotype was observed in 32 of the 35 heterozygous carriers of the HbS allele (10). As the HbS allele is not observed in ancestral Southeast Asian settlers of Madagascar, haplotypes associated with the HbA allele were further evaluated. Distinctly African HbA haplotypes were observed in the Malagasy samples studied at frequencies observed in African and African-American reference groups. In contrast, frequencies of distinctly Asian or Oceania HbA haplotypes were reduced between 41.4 and 52.8% (10). This study suggests significant admixture of African HbS and HbA allelic polymorphism within the Malagasy study population.

In the current study focused on Duffy blood group polymorphism we are able to perform comparisons similar to those made by Hewitt et al. between alleles considered to be African ($FY*B^{ES}$ and $FY*B$). In the general survey inclusive of 661 individuals (1,322 alleles) the African $FY*B^{ES}$ allele was observed at a frequency of 83.2%, $FY*A$ (of likely Asian origin) was found at 13.5%, and $FY*B$ at 3.3%. These results show that the Duffy-negative $FY*B^{ES}$ allele is present at frequencies higher than those of other African alleles studied to date (8–10). Whether this shows evidence of a selective advantage against *P. vivax* malaria in Madagascar will require further studies.

Appendix B: Duffy Blood Group Polymorphism, Working Nomenclature, and Function

The Duffy blood group antigen [Table S1: Duffy (Fy) nomenclature] was first observed in 1950 on erythrocytes using allo-antisera found in a multiply transfused hemophiliac (blood group namesake) who experienced a hemolytic transfusion reaction (11). The expected Fy^b antisera were discovered shortly thereafter in surveys of British populations; codominantly expressed Fy^a and Fy^b antigens were observed at frequencies of 41 and 59%, respectively. Upon screening African-American donors as in the Knickerbocker Blood Bank (New York), Sanger and colleagues found that 68% of the samples reacted with neither Fy^a nor Fy^b antisera and were temporarily classified as Duffy negative (12). Understanding difficulties of identifying an "Fy^c" antigen would require modern tools of molecular biology.

With the advent of molecular biology the gene sequence encoding the Duffy antigen (13) was shown to share homology with the family of seven-transmembrane g protein-coupled chemokine receptors, alternatively named Duffy antigen receptor for chemokines (DARC) (function discussed below). Further DNA sequence analysis of the Duffy antigen gene identified a single-nucleotide polymorphism (SNP) in a GATA-1 transcription factor binding site in the Duffy gene promoter (T → C at promoter position –33) (14). Subsequent gene expression analysis showed that this SNP blocks erythroid lineage expression of the Duffy antigen specifically (14), whereas the protein is expressed normally in endothelial cells of postcapillary venules (15). Working nomenclature has given this an "erythrocyte silent" (ES) designation. In individuals who are heterozygous carriers of a Duffy-negative allele, overall expression of the Duffy antigen on the erythrocyte surface is generally 50% reduced from levels observed for individuals homozygous for the wild-type –33T, Duffy-positive allele (16, 17). Duffy-negative African-Americans and Africans from the equatorial tropics through southern Africa are homozygous for this mutation (17–19) and in these individuals the –33C allele is upstream from the ORF sequence that would otherwise encode Fy^b, allele designation $FY*B^{ES}$. Homozygosity for the GATA-1 mutation drops to ≈50% along a cline from northern Africa onto the Arabian Peninsula. More recently the same Duffy –33C promoter SNP was identified in a Pv-endemic region of Papua New Guinea (17); however, in Papua New Guinea this SNP is upstream from the ORF sequence that would otherwise encode Fy^a, observed throughout Southeast Asia and Melanesia, allele designation $FY*A^{ES}$.

In addition to the GATA-1 promoter mutation, two additional SNPs occurring at polymorphic frequency (>1%) in the Duffy gene ORF are significant and influence Duffy antigen serological and expression phenotypes.At codon 44 a G → A transition leads to a Gly → Asp (G_G_T → G_A_T) amino acid substitution in the extracellular amino terminal domain and is responsible for the Fy^a vs. Fy^b antigens, respectively (20–22). Serological surveys have characterized most European populations to exhibit relatively equal frequencies for the Fy^a and Fy^b antigens, whereas Asian populations consistently exhibit higher Fy^a compared to Fy^b frequencies. In Melanesians, the frequency of the Fy(a+b–) phenotype ranges from 85 to 100% (17–19). At codon 89 a C → T transition causes an Arg → Cys (CGC → TGC) amino acid substitution within the first intracellular loop of the Duffy protein and is associated with the Fy^b and Fy^{bweak} antigens, respectively (23–25). The basis for the "weak" phenotype designation is that antibody and chemokine binding has been observed to be reduced by ≈90% in association with Fy^{bweak} vs. Fy^b antigens when analyzed by flow cytometry (25); the allelic designation corresponding to the Fy^{bweak} antigen is $FY*X$. The frequency of the $FY*X$ allele is ≈2% in Caucasians (23, 26); this SNP has not been observed in association with the $FY*A$ allele.

The overall Duffy phenotype is dependent upon both promoter and coding region SNPs. Expression phenotypes relative to 16 different genotypes possible from the five known Duffy alleles ($FY*A$, $FY*B$, $FY*X$, $FY*A^{ES}$, and $FY*B^{ES}$) are summarized in Table S1.

Duffy Antigen Function. The Duffy blood group antigen is a "silent" seven-transmembrane receptor. This results from the absence of a DRYLAIV amino acid motif in the second intracellular loop needed to couple with G proteins that initiate intracellular signaling cascades (27). Duffy is one of a few chemokine receptors that bind to inflammatory chemokines categorized by structural features into two different groups, α (amino acid motif –CC–) and β (amino acid motif –CXC–). On erythrocytes, the Duffy antigen is proposed to act as a sink that binds to excess chemokines and limits inflammation (28). Reciprocally, Duffy binding of chemokines prevents their diffusion into organs and peripheral tissue space and in this way acts as a reservoir of chemokines in the circulating blood (29). Duffy is also

expressed on a variety of nonerythroid cells including venular endothelial cells; in this context recent studies suggest two potential roles for Duffy. On venular endothelial cells Duffy has been proposed to act as a chemokine *inter*nalization re*ceptor* (interceptor) by internalizing and scavenging of chemokines (30). Alternatively, Pruenster et al. have shown that Duffy acts to mediate chemokine transcytosis (31). In their in vitro system, Duffy-mediated chemokine transcytosis led to apical retention of intact chemokines and leukocyte migration across Duffy-expressing endothelial cell monolayers. How these complex roles of the Duffy antigen are regulated remains to be determined.

Appendix C: Molecular Diagnostic Assays—Primers, Probes, and Reaction Conditions

Duffy Genotyping. Duffy genotyping included the GATA-1 transcription factor binding site at nucleotide position −33 (t, wild type; c, erythrocyte silent), the Fy^a/Fy^b antigen site at codon 42 (ggt encodes G, Fy^a; gat encodes D, Fy^b), and the Fy^{bweak} antigen site at codon 89 (cgc encodes R, Fy^b; tgc encodes C, Fy^{bweak}).

Direct sequencing. Primary PCR amplifications were performed in a reaction mixture (55 μL) containing 3 μL DNA, 0.4 μM each primer (forward primer, 5′-GTGGGGTAAGGCTTCCTGAT-3′; reverse primer, 5′-CAGAGCTGCGAGTGCTACCT-3′), 250 μM each dNTP, 2.5 mM $MgCl_2$, and 1.25 units TaKaRa DNA Polymerase (Ex Taq; Takara Bio Inc.) under the following conditions: 94 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min (PCR product 997 bp).

Nested PCR amplifications for SNP detection in the GATA-1 transcription factor binding site (forward primer, 5′-GTGGGGTAAGGCTTCCTGAT-3′; reverse primer, 5′-CAAACAGCAGGGGAAATGAG-3′) and exon 2 codon region (forward primer, 5′-CTTCCGGTGTAACTCTGATGG-3′; reverse primer, 5′-CAGAGCTGCGAGTGCTACCT-3′) were performed in separate reaction mixtures (55 μL) with 3 μL of PCR products, 0.36 μM each primer, 250 μM each dNTP, 2.5 mM $MgCl_2$, and 1.25 units TaKaRa DNA Polymerase following the amplification conditions provided above for 30 cycles [PCR products, 223 bp (GATA-1) and 402 bp (exon 2)].

After purification by filtration using a Macherey-Nagel plate (NucleoFast 96 PCR; Macherey-Nagel), sequencing reactions were performed for both strands using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit run on a 3730 xl Genetic Analyzer (Applied Biosystems). Electrophoregrams were visualized and analyzed with CEQ2000 Genetic Analysis System software (Beckman Coulter). Nucleotide sequences were compared to the glycoprotein D, Duffy group antigen sequence (GenBank accession no. S76830).

Post-PCR LDR-FMA. All post-PCR LDR-FMA methods include the same basic three-step procedure: (*i*) ligation of specific oligonucleotides to target single- or multiple-nucleotide polymorphisms, (*ii*) FlexMAP microsphere and streptavidin-R-phycoerythrin (SA:PE) labeling of sequence-specific ligation products, and (*iii*) detection of the specific fluorescent signals using the BioPlex suspension array system and Bio-Plex Manager analytical software (Bio-Rad Laboratories). These procedures have been described in detail for a variety of additional studies (32–34).

PCR was performed in a reaction mixture (28 μL) with 3 μL of PCR genomic DNA, 0.1 μM each primer (forward primer, Duffy-200up 5′-CAGGCAGTGGGCGTGGG-3′; reverse primer, Duffy +730dn 5′-CTGCTAGCTAGGATACCCAG-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, and 2.5 units of thermostable DNA polymerase under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 5 min (PCR products 912 and 1,033 bp).

Following PCR amplification, products were further processed by a ligation detection reaction (LDR). This LDR was performed in a reaction mixture (15 μL) containing 20 mM Tris-HCl buffer (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 1 mM NAD^+, 10 mM DTT, 0.1% Triton X-100, 13 nM each LDR probe, 1 μL of PCR product, and 2 units of Taq DNA ligase (New England BioLabs). LDR probes consisted of six allele-specific oligonucleotides and three fluorescently labeled conserved-sequence oligonucleotides. The allele-specific probes contained a TAG sequence for further hybridization with complementary sequence oligonucleotides bound to Luminex FlexMAP fluorescent microspheres. The conserved-sequence probes were phosphorylated at the 5′ end and biotinylated at the 3′ end.

Sequences of the probes used were as follows:

GATA-1 transcription factor binding site (-33T→C):

PRO T tag30 new: 5′-TTACCTTTATACCTTTCTTTTTAC-cattagtccttggctcttat-3′

PRO C tag 37: 5′-CTTTTCATCTTTTCATCTTTCAATtcat-tagtccttggctcttac-3′

PRO common: 5′-phosphate-cttggaagcacaggcgctg-biotin-3′.

Codon 42 encoding either the Fy^a antigen or the Fy^b antigen:

ORF G tag 12: 5′-TACACTTTCTTTCTTTCTTTCTTTttcc-cagatggagactatgg-3′

ORF A tag 28: 5′-CTACAAACAAACAAACATTAT-CAActtcccagatggagactatga-3′

ORF common: 5′-phosphate-tgccaacctggaagca-biotin-3′.

Reaction mixtures were initially heated for 2 min at 95 °C, followed by 32 cycles of 95 °C for 15 s and 58 °C for 2 min (annealing and ligation). The LDR product (5 μL) was then added to 60 μL of hybridization solution [3 M tetramethylammonium chloride (TMAC), 50 mM Tris-HCl (pH 8.0), 3 mM EDTA (pH 8.0), 0.10% SDS] containing 250 Luminex FlexMAP microspheres from each SNP-specific set (total number of SNP-specific microspheres, n = 6). Mixtures were heated to 95 °C for 90 s and incubated at 37 °C for 40 min to allow hybridization between SNP-specific LDR products and microsphere-specific anti-TAG oligonucleotides.

Following hybridization, 6 μL of streptavidin-R-phycoerythrin (Molecular Probes) in TMAC hybridization solution (20 ng/μL) was added to the post-LDR mixture and incubated at 37 °C for 40 min in Costar 6511 M polycarbonate 96-well V-bottom plates (Corning). Hybrid complexes consisting of SNP-specific LDR products and microsphere-labeled anti-TAG probes were detected using a Bio-Plex array reader (Bio-Rad Laboratories); the plate temperature was set to 37 °C throughout detection. All fluorescence data were collected using Bio-Rad software, Bio-Plex Manager 5.0.

Plasmodium* Species Diagnosis. Plasmodium *species small-subunit ribosomal DNA, post-PCR LDR-FMA assay. The assay used has been previously described (33). Except from an increase of the number of PCR cycles (up to 45 cycles), protocols used were similar for PCR, LDR, and preparation for processing by the Bio-Plex array reader and Bio-Plex Manager 5.0 software.

Plasmodium *species cytochrome oxidase subunit I (COI).* Primary PCR amplifications were performed in a reaction mixture (28 μL) containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-TCATTTTTATTTGGTAGTTATG-3′; reverse primer, 5′-CAAATCATATTCCATCCATTTA-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, and 2.5 units of thermostable DNA polymerase under the following conditions: 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 4 min (PCR product 1,288 bp). Nested PCR amplifications (972 bp) were performed as above using the forward primer, 5′-GATGGACTTTATATCCACCAT-3′.

Following PCR amplification, products were further processed by LDR as indicated above in the method provided for Duffy genotyping. Specific LDR probes consisted of five species-specific probes and three fluorescently labeled conserved-sequence probes. The conserved-sequence probes were phosphorylated at the 5′ end and labeled with biotin at the 3′ end. The allele-specific probes contained a Tag sequence for further analysis and linking with fluorescent beads.

Sequences of the probes used for detection of *Plasmodium* species were as follows:

COI *P. species* tag 3: 5′-tacactttatcaaatcttacaatcGATTTAATGTAATGCCTAGA
COI *P. falciparum* tag 5: 5′-caattcaaatcacaataatcaatcGATTATTTACAACYGTAAGTGCA-3′
COI *P. vivax* tag 59: 5′-tcatcaatcaatctttttcactttGATTGTTTACATTAGTAAGTAGT-3′
COI *P. malariae* tag 8: 5′-aatccttttacattcattacttacGATTATTTACTACAATAAGTCAT-3′
COI *P. ovale* tag 62: 5′-tcaatcataatctcataatccaat GATTATTTACAACAGTAAGTGCT-3′
COI *P. species* common 1: 5′-phosphate-TTTCAAGAAAACTTTTTTGG-biotin-3′
COI *P. species* common 2: 5′-phosphate-TTTCAAGATAATTTCTTTGG-biotin-3′
COI *P. species* common 3: 5′-phosphate-CGTATTCCTGATTATCCAGA-biotin-3′.

Reaction mixtures were initially heated for 1 min at 95 °C, followed by 32 cycles of 95 °C for 15 s and 58 °C for 2 min (annealing and ligation). The LDR product (5 μL) was then added to 60 μL of hybridization solution [3 M TMAC, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA (pH 8.0), 0.10% SDS] containing 250 Luminex FlexMAP microspheres for each sequence-specific set. Mixtures were further processed for detection as indicated previously in methods provided for Duffy promoter and coding region SNPs.

Real-time PCR (35). Plasmodium species were detected by real-time PCR with a RotorGene 3000 thermocycler (Corbett Life Science); PCRs were performed in 25 μL reaction mixture contained 2.5 μL of sample DNA, 12.5 μL of qPCR MasterMix Plus for SYBR Green I No ROX (Eurogentec), and 0.6 mM of each primer (PL1473F18, 5′-TAACgAACgAgATCTTAA-3′; PL1679R18, 5′-gTTCCTCTAAgAAgCTTT-3′). PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by amplification for 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 90 s at 65 °C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melting program consisting of 2 min at 95 °C, 2 min at 68 °C, and a stepwise temperature increase of 0.5 °C/s up to 90 °C, with fluorescence acquisition at each temperature transition. The fluorescence data were analyzed using F1/F2 settings, which improved the detection of *P. falciparum*, and a cutoff of 40 cycles was used to define *Plasmodium*-positive samples.

Classical species-specific nested PCR targeting Plasmodium sp. small subunit ribosomal RNA (36). Outer PCR amplifications were performed in 50 μL reaction mixture containing 3 μL DNA, 0.6 μM each primer (rPLU5, 5′-CCTGTTGTTGCCTTAAA-CTTC-3′; rPLU6, 5′-TTAAAATTGTTGCAGTTAAAACG-3′), 200 μM each dNTP, 2.5 mM $MgCl_2$, and 1.25 units TaKaRa DNA Polymerase (Ex Taq; Takara Bio Inc.) under the following conditions: first cycle heating at 95 °C for 8 min, hybridization at 58 °C for 60 s, extension at 72°C for 120 s, followed by 38 cycles of 95 °C for 60 s, 58 °C for 60 s, and 72 °C for 180 s and a final cycle of 95 °C for 60 s, 58 °C for 60 s, and 72 °C for 15 min. Inner PCR amplifications were performed under the same conditions by using the following specific primers for each species: rFAL1, 5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′, and rFAL2, 5′-ACACAATGAACTCAATCATGACTACCCGTC-3′, for *P. falciparum*; rVIV1, 5′-CGCTTCTAGCTTAATCCACATAACTGATAC-3′, and rVIV2, 5′ ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3′, for *P. vivax*; rMAL1, 5′-ATAACATAGTTGTACGTTAAGAATAACCGC-3′, and rMAL2, 5′ AAAATTCCCATGCATAAAAAATTATACAAA-3′, for *P. malariae*; and rOVA1, 5′-ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3′, and rOVA2, 5′-GGAAAAGGACACATTAATTGTATCCTAGTG-3′, for *P. ovale.*

The PCR products of nested amplifications were analyzed by gel electrophoresis and staining with ethidium bromide. The sizes of the amplified DNA fragments were 1,000, 205, 144, 120, and 788 bp for genus *Plasmodium*, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, respectively.

Plasmodium vivax* Strain Analyses.** ***P. vivax circumsporozoite protein repeat polymorphism (37). Primary PCR amplifications were performed in a reaction mixture (28 μL) containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-ATGTAGATCTGTCCAAGGCCATAAA-3′; reverse primer, 5′-TAATTGAATAATGCTAGGACTAACAATATG-3′), 180 μM each dNTP, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 50 mM KCl, and 2.5 units of thermostable DNA polymerase under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 4 min.

Nested 2-PCR amplifications were performed using a similar reaction mixture (28 μL) with 3 μL of PCR products, 0.1 μM each primer (forward primer, 5′-GCAGAACCAAAAAATCCACGTGAAAATAAG-3′; reverse primer, 5′-CCAACGGTAGCTCTAACTTTATCTAGGTAT-3′), under the following conditions: heating at 95 °C for 2 min, followed by 40 cycles of heating at 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 4 min. Seminested 3-PCR amplifications were performed using a similar 28-μL reaction mixture with 3 μL of PCR products from nested 2 PCR, 0.1 μM each primer (forward primer specific for *P. vivax* CSP subtypes, VK210, 5′-GGHGATAGAGCAGCTGGAC-3′, and VK247, 5′-GCAAATGGGGCHGGYAATC-3′; reverse primer was the same as the one used for nested 2 PCR), under the following conditions: heating at 95 °C for 2 min, followed by 40 cycles of heating at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 4 min.

Following PCR amplification, products were further processed by LDR as indicated above in the method provided for Duffy genotyping. Specific LDR probes consisted of two allele-specific probes and two fluorescently labeled conserved-sequence probes. The conserved-sequence probes were phosphorylated at the 5′ end and labeled with biotin at the 3′ end. The allele-specific probes contained a Tag sequence for further analysis and linking with fluorescent beads.

Sequences of the probes used were as follows:

P. vivax subtype VK210 isolates:
VK210tag28, 5′-ctacaaacaaacaaacattatcaaGGAGATAGAGCAGCTGGACAGCCAGCW-3′
ConservedVK210, 5′-phosphate-GGAAATGGTGCAGGTGGACAGGCAGCAG-biotin-3′.
P. vivax subtype VK247 isolates:
VK247tag80, 5′-ctaactaacaataatctaactaacGCAAATGGGGGCAGGTAATCAAGGA-3′
ConservedVK247, 5′-phosphate-GCAAATGGTGCAGGTGGACAGGCAGCA-biotin-3′.

The reaction mixtures were initially heated for 1 min at 95 °C, followed by 32 thermal cycles of 95 °C for 15 s (denaturation)

and 58 °C for 2 min (annealing and ligation). The LDR product (5 μL) was then added to 60 μL of hybridization solution [3 M TMAC, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA (pH 8.0), 0.10% SDS] containing 250 Luminex FlexMAP microspheres from each allelic set (total number of alleles, two). Mixtures were further processed for detection as indicated previously in methods provided for Duffy promoter and coding region SNPs.

Duffy binding protein (38). Primary PCR amplifications were performed in 28 μL reaction mixture containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-AATAATACAGACACAAATTTTCAT-3′; reverse primer, 5′-ATAAGGAGTTACGATACCTGC-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, and 2.5 units of thermostable DNA polymerase under the following conditions: 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 90 s and a final extension at 72 °C for 5 min (PCR product 663 bp).

Nested PCR applications were performed using a similar reaction mixture (28 μL) with 3 μL of PCR products, 0.1 μM each primer (forward primer, 5′-CTTATTTATGATGCTGCAGTAGAG-3′; reverse primer, 5′-TTGACATGGTGGTACCTTACATAC-3′), under the following conditions: 95 °C for 1 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 5 min (PCR product 474 bp). Positivity of the amplification was checked on a 2% agarose gel with SybrGold staining.

P. vivax genotyping using microsatellite markers (39–41). *P. vivax* genotyping was performed by using six microsatellite markers (2.21, 14.185, 8.332, L40, 6.34, and L34), previously described as polymorphic. Microsatellite PCR products were genotyped on the basis of size, using a GeneScan 500 LIZ size standard on an ABI Prism 3730 XL DNA analyzer.

Primary PCR analyses for the six microsatellite markers were done in a total volume of 20 μL that contained 0.1 μmol/L of each primer (2.21, Pv2.21-PF 5′-GGCAGGAACGTAGAGGAG-3′ and Pv2.21-PR 5′-GGCTTGTTCATTTTGAGGTA-3′; 14.185, Pv14.185-PF 5′-TGCAGATATGCTGTCGAAT-3′ and Pv14.185-PR 5′-GGGAAAAACTTGGTCACAC-3′; 8.332, Pv8.332-PF 5′-TGAAGCAATATAGCGATGAC-3′ and Pv8.332-PR 5′-CGGTGTAGTGTGGTACAATG-3′; L40, PvL40-PF 5′-ATTTGTGTATGCCTTGTGTT-3′ and PvL40-PR 5′-GTGAGGGTGTCTATCCGTA-3′; 6.34, Pv6.34-PF 5′-CCCAATTAAGTGCAAATCA-5′ and Pv6.34-PR 5′-CATGTAAAGAGGCACATGG-3′; and L34, PvL34-PF 5′-TACCCCAGCCTTATCTCTC-3′ and PvL34-PR 5′-AAATGCACAGACACTACGC-3′), 200 μmol/L of dNTPs, 2.5 mmol/L of $MgCl_2$, 1.25 units TaKaRa DNA Polymerase (Ex Taq; Takara Bio Inc.), and 2 μL of genomic DNA. Two microliters of undiluted PCR product was used as a template for the nested PCR in a volume of 20 μL with 0.5 μmol/L of each specific primer labeled with a fluorescent dye (2.21, Pv2.21-NF 5′-6FAM-CCATCTGCTCAAATCCGAAG-3′ and Pv2.21-NR 5′-GGCTCCTCCCTGTCTCTGTAG-3′; 14.185, Pv14.185-NF 5′-6FAM-GCAGTTGTTGCAGATTGAGC-3′ and Pv14.185-NR 5′-TAAGGCGTGCACGTTATCAT-3′; 8.332, Pv8.332-NF 5′-HEX-CCTCGATGGTGATGTGATGA-3′ and Pv8.332-NR 5′-GTATAACATGGCACCCGACCT-3′; L40, PvL40-NF 5′-HEX-GTTTACCAGGCCCAATTCAC-3′ and PvL40-NR 5′-GTTCACACGGGCGTATACAT-3′; 6.34, Pv6.34-NF 5′-6FAM-TGAGCGCTTTAAGCTTCTGC-3′ and Pv6.34-NR 5′-CAAAAATGAATCGTGGCACA-3′; and L34, PvL34-NF 5′-6FAM-TTTTCCCTTCGGAAAAACG-3′ and PvL34-NR 5′-ACGACCATCACCTGCCATAG-3′), using the same conditions as for primary PCR.

PCR analyses were performed under the following conditions: initial denaturation for 4 min at 94 °C; 30 cycles (primary PCRs) or 45 cycles (nested PCRs) of denaturation for 20 s at 94 °C, annealing for 20 s at 57 °C (primary PCRs) or 60.5 °C (nested PCR for L40), 61 °C (nested PCR for 14.185), 62 °C (nested PCR for 2.21 and L34), and 62.5 °C (nested PCR for 8.332 and 6.34); elongation for 30 s at 72 °C; and final elongation for 10 min at 72 °C. PCR products were first analyzed on a 2% agarose gel. For capillary electrophoresis, most nested PCR products were not diluted or diluted to 1:20. A total of 2.5 μL of PCR product was mixed with 10 μL of diluted size standard GeneFlo 625 DNA ladder, Rox-labeled (EURx Molecular Biology Products). After 1 h of incubation at room temperature, the samples were run on a 3730xls DNA analyzer (Applied Biosystems). Data were analyzed using GeneMapper version 4.0 (Applied Biosystems), to facilitate determination of fragment sizes and peak intensity. All samples were checked visually and alleles were grouped manually according to their size and to their repeat length for microsatellites.

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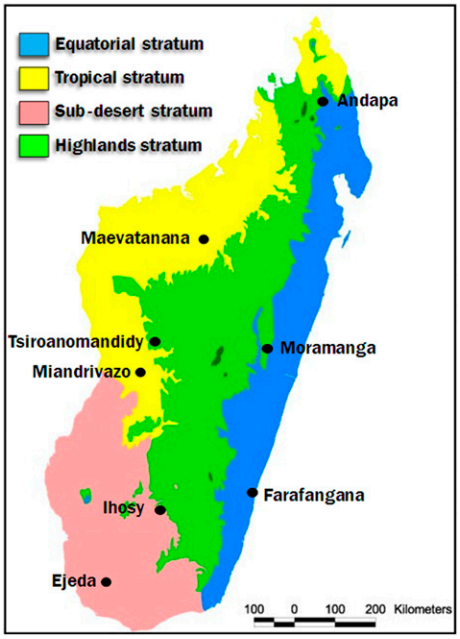

Fig. S1. Study sites and their distribution throughout Madagascar's four malaria ecological strata.

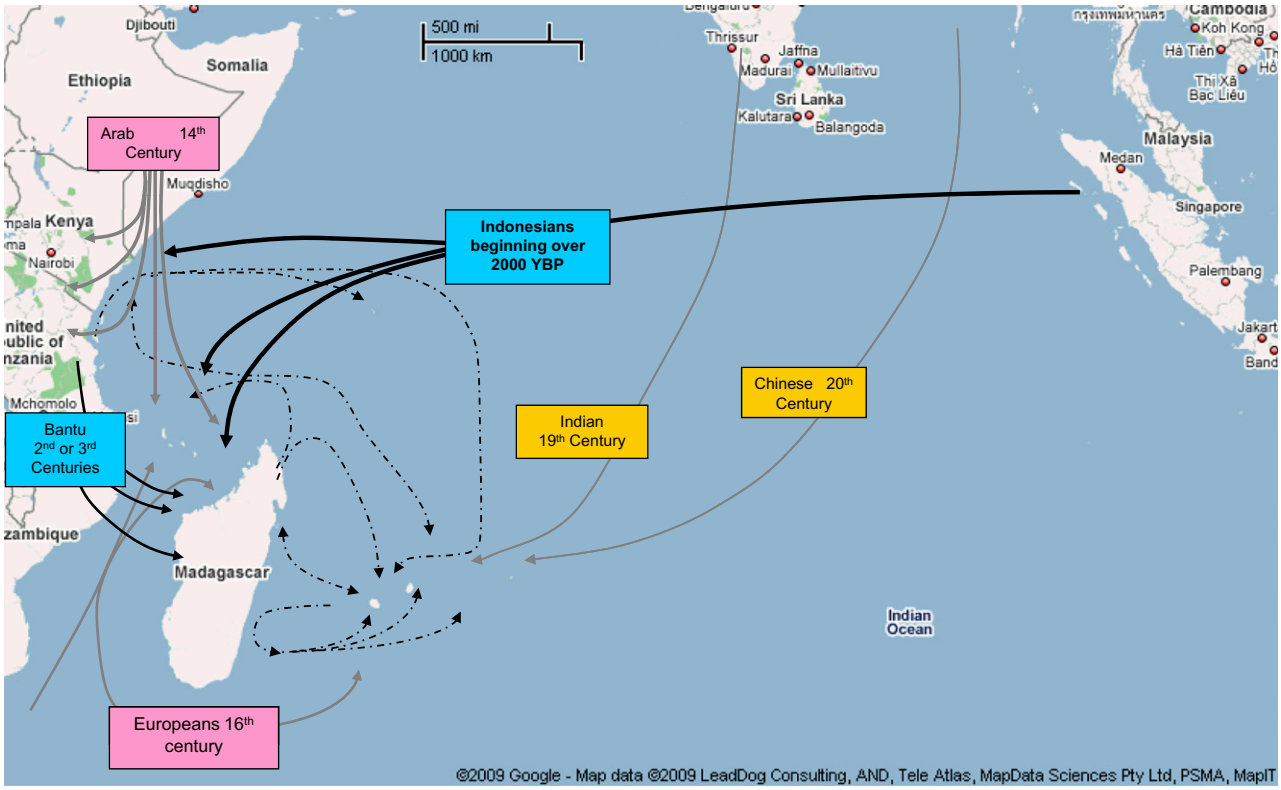

Fig. S2. Peopling history of Madagascar and the Indian Ocean trade network (adapted from ref. 1).

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Table S1. Working guidelines for Duffy* blood group nomenclature[†]

Allele[‡]	Antigen	Genotype	Phenotype	
			Serological	Expression[§]
*FY*A*	Fy^a	*FY*A/FY*A*	Fya+/b−	2× Fy^a, 0× Fy^b
*FY*B*	Fy^b	*FY*A/FY*X*	Fya+/b−	1× Fy^a, 0.1× Fy^b
*FY*X*	Fy^{bweak}	*FY*A/FY*A^{ES}*	Fya+/b−	1× Fy^a, 0× Fy^b
FYA^{ES}*	No antigen	*FY*A/FY*B^{ES}*	Fya+/b−	1× Fy^a, 0× Fy^b
FYB^{ES}*	No antigen	*FY*B/FY*B*	Fya−/b+	0× Fy^a, 2× Fy^b
		*FY*B/FY*X*	Fya−/b+	0× Fy^a, 1.1× Fy^b
		*FY*B/FY*A^{ES}*	Fya−/b+	0× Fy^a, 1× Fy^b
		*FY*B/FY*B^{ES}*	Fya−/b+	0× Fy^a, 1× Fy^b
		*FY*X/FY*X*	Fya−/b+^{weak}	0× Fy^a, 0.2× Fy^b
		*FY*X/FY*A^{ES}*	Fya−/b+^{weak}	0× Fy^a, 0.1× Fy^b
		*FY*X/FY*B^{ES}*	Fya−/b+^{weak}	0× Fy^a, 0.1× Fy^b
		*FY*A/FY*B*	Fya+/b+	1× Fy^a, 1× Fy^b
		*FY*A/FY*X*	Fya+/b+^{weak}	1× Fy^a, 0.1× Fy^b
		FYA^{ES}/FY*A^{ES}*	Fya−/b−	0× Fy^a, 0× Fy^b
		FYA^{ES}/FY*B^{ES}*	Fya−/b−	0× Fy^a, 0× Fy^b
		FYB^{ES}/FY*B^{ES}*	Fya−/b−	0× Fy^a, 0× Fy^b

Alleles correspond with antigens. Genotypes (allele combinations) correspond with phenotypes.
*Alternate gene name: Duffy antigen/receptor for chemokines (*DARC*).
[†]Consistent with the blood group mutations database at the National Center for Biotechnology Information, official nomenclature to be determined.
[‡]ES, erythrocyte silent, attributed to a T to C transition at nucleotide −33 in the Duffy gene promoter.
[§]Expression phenotypes based on a composite of flow cytometry and chemokine binding (references documenting expression phenotypes are provided in the *SI Text*).

Table S2. Duffy* phenotype/genotype and *Plasmodium* infections in Malagasy schoolchildren

Duffy Phenotype	Fy(a+b−)		Fy(a+b+):	Fy(a−b+)		Fy(a−b−):	
Duffy Genotype	*FY*A/*A*	*FY*A/*B^{ES}*	*FY*A/*B*	*FY*B/*B*	*FY*B/*B^{ES}*	*FY*B^{ES}/*B^{ES}*	Totals
Total population	25 (3.8%)	117 (17.7%)	11 (1.7%)	1 (0.1%)	31 (4.7%)	476 (72.0%)	661
Pv infection (prevalence)[†]	2 (8.0%)	33 (28.2%)	5 (45.5%)	—	4 (12.9%)	42 (8.8%)	86 (13.0%)
Plasmodium sp. infection (prevalence)[‡]	12 (48.0%)	45 (38.5%)	6 (54.5%)	—	6 (19.4%)	121 (26.7%)	190 (28.7%)
Study site[§]							
Andapa: Duffy genotype	1	3	0	0	1	59	64
Pv infection	—	—	—	—	—	—	—
Plasmodium sp. infection	—	—	—	—	—	14	14
Farafangana: Duffy genotype	2	9	0	0	1	74	86
Pv infection	—	—	—	—	—	1	1
Plasmodium sp. infection	—	1	—	—	—	18	19
Maevatanana: Duffy genotype	2	20	1	0	3	72	98
Pv infection	—	2	—	—	—	2	4
Plasmodium sp. infection	1	3	—	—	—	12	16
Miandrivazo: Duffy genotype	8	18	0	0	2	60	88
Pv infection	—	5	—	—	—	9	14
Plasmodium sp. infection	5	9	—	—	1	21	36
Moramanga: Duffy genotype	2	15	1	1	7	43	69
Pv infection	—	2	—	—	—	—	2
Plasmodium sp. infection	—	2	—	—	—	8	10
Tsiroanomandidy: Duffy genotype	5	33	6	0	7	57	108
Pv infection	2	24	5	—	4	30	65
Plasmodium sp. infection	5	29	6	—	5	42	87
Ejeda: Duffy genotype	1	8	1	0	5	42	57
Pv infection	—	—	—	—	—	—	—
Plasmodium sp infection	—	—	—	—	—	1	1
Ihosy: Duffy genotype	4	11	2	0	5	69	91
Pv infection	—	—	—	—	—	—	—
Plasmodium sp. infection	1	1	-	—	—	5	7

*Duffy working nomenclature is reviewed in *SI Appendix B*.
[†]Pv infection is any *Plasmodium* species infection including *P. vivax*.
[‡]*Plasmodium* sp. infection is any *Plasmodium* species-positive individuals.
[§]Eight study sites, 2006–2007; location, climate, malaria endemicity, and ethnic distribution are reviewed in *SI Appendix A*.

Table S3. Duffy* phenotype/genotype in individuals experiencing clinical malaria[†]

Duffy Phenotype	Fy(a+b−)		Fy(a+b+)		Fy(a−b+)			Fy(a−b−):	
Duffy Genotype	*FY*A/*A*	*FY*A/*B^{ES}*	*FY*A/*B*	*FY*A/*X*	*FY*B/*B^{ES}*	*FY*B/*X*	*FY*X/*B^{ES}*	*FY*B^{ES}/*B^{ES}*	Totals
Patient population	21 (11.5%)	95 (51.9%)	6 (3.3%)	7 (3.8%)	20 (10.9%)	4 (2.2%)	13 (7.1%)	17 (9.3%)	183
Pure Pv infection	20	84	5	6	20	4	5	9	153
Mixed Pv/Pf infection[‡]	1	11	1	1	—	—	8	8	30
Study site[§]									
Maevatanana: Duffy genotype	1	9	1	3	1	0	2	1	18
Pure Pv infection	1	9	1	3	1	—	2	1	18
Mixed Pv/Pf infection	—	—	—	—	—	—	—	—	—
Tsiroanomandidy: Duffy genotype	12	49	3	3	11	3	10	7	98
Pure Pv infection	11	38	2	2	11	3	3	1	71
Mixed Pv/Pf infection	1	11	1	1	—	—	7	6	27
Miandrivazo: Duffy genotype	7	29	1	1	8	0	1	7	54
Pure Pv infection	7	29	1	1	8	—	—	6	52
Mixed Pv/Pf infection	—	—	—	—	—	—	1	1	2
Moramanga: Duffy genotype	0	2	0	0	0	1	0	1	4
Pure Pv infection	—	2	—	—	—	1	—	1	4
Mixed Pv/Pf infection	—	—	—	—	—	—	—	—	—
Ihosy: Duffy genotype	0	1	0	0	0	0	0	1	2
Pure Pv infection	—	1	—	—	—	—	—	—	1
Mixed Pv/Pf infection	—	—	—	—	—	—	—	1	1
Ejeda: Duffy genotype	1	5	1	0	0	0	0	0	7
Pure Pv infection	1	5	1	—	—	—	—	—	7
Mixed Pv/Pf infection	—	—	—	—	—	—	—	—	—

*Duffy working nomenclature is reviewed in *SI Appendix B*. Only those Duffy genotypes/phenotypes associated with *Plasmodium* species infections during the in vivo efficacy studies testing for *P. vivax* drug resistance are included in this table.

[†]Clinical *P. vivax* malaria was based on fever (≥37.5 °C) within 48 h of health center visit and positive rapid diagnostic test (RDT). As RDT was not specific for *P. vivax*, the species attributed to disease was determined by microscopy (*P. vivax* parasitemia >250 infected erythrocytes/μL) and PCR-positive confirmation.

[‡]All *Plasmodium* species confirmed by molecular diagnosis

[§]Eight study sites, 2006–2007 (no *P. vivax* infection was observed in Andapa and Farafangana).