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(a−b−)) 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 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.

Duffy | 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 Fyα nor Fyβ 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 Fyα and Fyβweak 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−); nomenclature descri-


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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. GU130196 and GU130197).

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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 subject 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*BES/*BES) and 28% (185/661) were Duffy positive (17.7% FY*A/*BES, 4.7% FY*B/*BES, 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 Tsiraoanomandidy \((n = 30)\) and Miandrivazo \((n = 9)\), study sites with the highest frequencies of Duffy-positive study participants \((47.2\) and \(31.8\)\%, respectively).

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 (Tsiraoanomandidy \(\chi^2 = 1.07, P = 0.30;\) Miandrivazo \(\chi^2 = 0.01, P = 0.92;\) Maevatanana \(\chi^2 = 0.31, P = 0.80;\) Farafangana \(\chi^2 = 0.08, P = 0.78\)). 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% \((14/153)\) 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 \((Fig. 2.4 and B)\) 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 Tsiraoanomandidy 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 FY6/Fy6 antigentic classifications. Fig. 3 illustrates flow cytometry phenotypes comparing control donors and field samples. Fig. 3A 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*A/*A, FY*BES/*BES, FY*A/*B, and FY*B/*B. Donors showed expected patterns of anti-Fy6 antibody. Additionally, Fig. 3A illustrates flow cytometry results for one FY*BES/*BES Malagasy donor were identical to the West African FY*BES/*BES controls \((Fig. 3.B–I)\) 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. 3B shows results for 40 Malagasy individuals, with 30 samples genotyped FY*B/*B, FY*B/*ES, FY*A/*B, FY*A/*ES, //Fy6 a−b−b−, 2 genotypes FY*A/*A FY[a−b−b−], and 2 genotypes FY*A/*B FY[a−b−b−]. Results show that Duffy antigen expression was uniformly absent from erythrocyte surfaces of all FY*B/*B, FY*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*E alleles and the FY*B*E*E allele.
zygosity (VK210 and VK247 variants (VK210, VK247) variants (VK210, VK247), detect the Duffy antigen by serology resulted from the 30-year-old female, genotyped as Duffy negative (FY*Bfy*Bfy*)), 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 parasites/μL) and P. falciparum (parasitemia = 980 parasites/μL). PCR-based Plasmodium species diagnosis confirmed the blood smear result; P. malariae and P. ovale were not detected. A shows an uninfected 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*Bfy*Bfy*), male, who presented at the Mandrivo health center (July 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 = 0.17, 3,040 parasites/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*Bfy*Bfy*) 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 parasites/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 (He) 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 (Fst = 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 Mandoiravo), 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*Bfy*Bfy*) 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.
P. vivax readily infects erythrocytes of the squirrel monkey (Saimiri boliviensis) (29, 30). Whereas squirrel monkeys express an Fy-negative Duffy antigen (29–31), the P. vivax DBP binds poorly, if at all, to squirrel monkey erythrocytes (32), suggesting a PDBP-independent invasion mechanism. In vitro studies showing that P. knowlesi invades Duffy-negative erythrocytes treated with trypsin and neuraminidase (33) suggest that P. knowlesi possesses 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*AF5) (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 PDBP 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/SANFFPS/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 Tsimanomandidy study population.

In vivo efficacy studies on antimalarial drugs were conducted in 2006 and 2007 at the eight study sites (registration no. IRSCN36517335) (26, 27). P. vivax clinical samples, collected on filter paper, were selected from all patients screened by a rapid diagnostic test (RDT) (Optimal-MAL-IT; Diamed AG). Giemsa-stained 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 an 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 (Arscam) (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 −123, Codon 42, Fy*Y-A vs. Fy*Y-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 clinical samples 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 monoclonal antibodies (BD) or monoclonal antibodies (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: Fy6 antibody (Fy* specific), HICR31 antibody (Fy* specific), and anti-Fy6 antibody (NaM185-2C3 clone, Duffy specific) (45). Briefly, erythrocytes from EDTA-anticoagulated fluid and control samples [Fy(a−b+), Fy(a−b−), and Fy(a−bweak) vs Control 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 IgG1Gm (5 μg/mL, BD) or monoclonal antibodies (anti-Fy6 diluted at 1:8, HICR31 and Fy6 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

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Fig. 3. Flow cytometry and serological correlation of Duffy-negative and Duffy-positive phenotypes with respective genotypes in Malagasy school children from Tsimaonamandavy, 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*A/*A FY*Y*6/Fy(a−b+) (red) and five well-characterized control donors who are FY*A/*B FY*Y*6/Fy(a−b+) (solid black), FY*Y*6/Fy(a−bweak) (light green), FY*Y*X/Fy(a−bweak) (green), FY*A/ Fy(a−b+) (light blue), and FY*AY*FY*6/Fy(a+B+) (blue), respectively. Results include fluorescence of a Duffy-negative 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*Y*6/Fy(a−b+) Malagasy samples (mean = 48, SD = 1,2), 6 FY*AY*6 FY*Y*6/Fy(a−b+) Malagasy samples (mean = 1,025, SD = 22,4), 2 FY*AY*FY*6/Fy(a+B+) Malagasy samples (mean = 1,937, SD = 54) and 2 FY*AY*FY*6/Fy(a+B+) Malagasy samples (mean = 1,896, SD = 8).

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acquired by a high-dimensional flow cytometry. Erythrocytes were identified on the basis of forward scatter characteristics, using log-likelihood discrimination. 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 estimated heterozygosity (Hₑ) from haploid data and calculated as Hₑ = [n(i−1)]/ [1 – Σn(i)] (n is the number of isolates sampled, pᵢ is the frequency of the Hₑ 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 km²) 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 Antemoros, 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 Zafy soro 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°43′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.

Mandrivazo—latitude 19°31′S, longitude 45°29′E. Mandrivazo, 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). 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.

Tsiranoanomidy—latitude 18°77′S, longitude 46°04′E. Tsiranoanomidy 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 Antsiranan. 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 Amanganja, 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 highland 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 Maayan 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; Betselelo, 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 (9). 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<sub>y</sub><sup>a</sup> 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, whereas 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 within the Malagasy study population. Distinctly African HbA haplotypes were observed in 32 of the 35 heterozygous carriers of the HbS allele (10). As the HbS allele is not observed in ancestral Southeast Asian Y chromosome haplotypes, two populations from Borneo are the best candidates for their likely origin. Whereas these results suggest a predominance of African paternal and Malaysian maternal ancestry, overall statistical analyses from these studies are inconclusive. F<sub>y</sub><sup>a</sup> 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, whereas it is lower in coastal populations.

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<sub>G</sub> → A<sub>A</sub>) amino acid substitution in the extracellular amino terminal domain and is responsible for the Fy<sup>a</sup> vs. Fy<sup>b</sup> antigens, respectively (20–22). Serological surveys have characterized most European populations to exhibit relatively equal frequencies for the Fy<sup>a</sup> and Fy<sup>b</sup> antigens, whereas Asian populations consistently exhibit higher Fy<sup>2</sup> compared to Fy<sup>b</sup> frequencies. In Melanesians, the frequency of the Fy<sup>(a+b−)</sup> 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 intracellular loop of the Duffy protein and is associated with the Fy<sup>a</sup> and Fy<sup>b</sup><sup>weak</sup> 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<sup>b</sup><sup>weak</sup> vs. Fy<sup>b</sup> antigens when analyzed by flow cytometry (25); the allelic designation corresponding to the Fy<sup>b</sup><sup>weak</sup> antigen is Fy<sup>∗</sup>X. The frequency of the Fy<sup>∗</sup>X allele is ~2% in Caucasians (23, 26); this SNP has not been observed in association with the Fy<sup>(a+b)</sup> 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<sup>a</sup>, Fy<sup>b</sup>, Fy<sup>∗</sup>X, Fy<sup>a</sup><sup>weak</sup>, and Fy<sup>b</sup><sup>weak</sup>) are summarized in Table S1.

**Duffy Antigen Function.** The Duffy blood group antigen is a “silent” seven-transmembrane receptor. This result stems from the absence of a DRYLAIIV 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 −CX−C−). 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 genetically related to the adhesion molecule selectin E (SELJE) and is one of the four chemokine C-type lectin receptors (CCLR). Duffy is also related to the circulating chemokine receptor CCR1, which is also considered a C-type lectin receptor (CCLR).

**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 name–sake) who experienced a hemolytic transfusion reaction (11). The expected Fy<sup>a</sup> antisera were discovered shortly thereafter in surveys of British populations; codominantly expressed Fy<sup>a</sup> and Fy<sup>b</sup> 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<sup>a</sup> nor Fy<sup>b</sup> antisera and were temporarily classified as Duffy negative (12). Understanding difficulties of identifying an “Fy<sup>c</sup>” 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<sup>a</sup>, allele designation Fy<sup>a</sup><sup>ES</sup>. 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 P- and V-enzyme region, allele designation Fy<sup>a</sup><sup>ES</sup>.

The Duffy blood group antigen is a seven-transmembrane receptor that binds to inflammatory chemokines categorized by structural features into two different groups, α (amino acid motif −CC−) and β (amino acid motif −CX−C−). 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 related to the circulating chemokine receptor CCR1, which is also considered a C-type lectin receptor (CCLR). Duffy is also related to the adhesion molecule selectin E (SELJE) and is one of the four chemokine C-type lectin receptors (CCLR). Duffy is also genetically related to the adhesion molecule selectin E (SELJE) and is one of the four chemokine C-type lectin receptors (CCLR).
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 internalization receptor (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\(^\alpha/FY\(^\beta\) antigen site at codon 42 (gtt encodes G, FY\(^\alpha\); gat encodes D, FY\(^\beta\)), and the FY\(^\beta\)weak antigen site at codon 89 (gcg encodes R, FY\(^\beta\); tgc encodes C, FY\(^\beta\)weak).

**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′-GGAGCTGCGAGTGCTACCT-3′; reverse primer, 5′-CAAGGCTCGAGGCTGACCT-3′) and 1.25 units TaKaRa DNA Polymerase (Ex Taq; Takara Bio Inc.) under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 51 °C for 30 s, and 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′-GGGTTGT-AAGGCTCTGCT-3′; reverse primer, 5′-CACAACAGCAGGGAAATGAG-3′) and exon 2 codon region (forward primer, 5′-CTTCCGGTTAACCTGATG-3′; reverse primer, 5′-CAGAGCTCTGAGGCTGACCT-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 (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).

**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-phycocerythrin (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). 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-phycocerythrin (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′-CAGGGAGTGGGCGTGGG-3′; reverse primer, Duffy + 738dn 5′-CTGCTAGCTAGGATACCAGG-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgSO\(_4\), 16.6 mM (NH\(_4\))\(_2\)SO\(_4\), 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 Lumixen 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** tag 30 new: 5′-TTACCTTTATACCTTCTTTTTAC-3′
- **PRO C** tag 37: 5′-CTTCTTATCTTTTCTTCTTTTAAATC-3′
- **PRO common:** 5′-phosphate-ctggaaagacagcctgcg-biotin-3′.

Codon 42 encoding either the FY\(^\alpha\) antigen or the FY\(^\beta\) antigen:

- **ORF G** tag 12: 5′-TACACTTCTTTCTTTTCTTTTAAATC-3′
- **ORF A** tag 28: 5′-CTCAAAACAAACACATCTTACACGATAGGATCTA-3′
- **ORF common:** 5′-phosphate-tgcaaccagctggaagcctg-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 containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.10% SDS containing 250 Lumixen 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-phycocerythrin (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 (35). 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′-TACTTTTTATTTTGTAGATTG-3′; reverse primer, 5′-CACAATCTATTTCCATCATTATTA-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgSO\(_4\), 16.6 mM (NH\(_4\))\(_2\)SO\(_4\), 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, 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′-GATGGGACTTATATCACCACAT-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. falciparum* tag 5: 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′
- *P. malariae* tag 8: 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′
- *P. ovale* tag 64: 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′
- *P. vivax* tag 35: 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′

**TAATTTCTTTGG-biotin-3′** for further analysis and linking with fluorescent beads.

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. 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′-ATGTAGATCTGTCC-AAGGCGCATATA-3′; reverse primer, 5′-TAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′), 180 μM each dNTP, 10 μM Tris-HCI (pH 8.0), 1.5 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 μM 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. Seminested 3-PCR amplifications were performed using a similar 28-μL reaction mixture with 3 μL of PCR products, 0.1 μM each primer (forward primer, 5′-CGCAAACAGAAATATACCCAG-TGAAAATAG-3′; reverse primer, 5′-CCACCGTAGCTGCTAACCTTATGTTGATTGTAC-3′), 180 μM each dNTP, 10 μM Tris-HCI (pH 8.0), 1.5 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 μM 2-mercaptoethanol, 50 mM KCl, and 2.5 units of thermostable DNA polymerase 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. 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′-TAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′

ConservedVK210, 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′

**P. vivax** subtype VK247 isolates:

VK247tag80, 5′-TAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-3′

ConservedVK247, 5′-TCAATTTTACACACTTGTCTGTTGGGTGG-TATTAGTATACTG-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 reaction mixture (28 μL) was formed in 28 μL reaction mixture containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-AATATAATACAGACACAAATTTTAC-3′; reverse primer, 5′-ATAAGGATCTACAGATCTTCG-3′), 180 μM each dNTP, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgSO4, 16.6 mM (NH4)2SO4, 10 mM 2-mercaptoethanol, and 2.5 units of thermostable DNA polymerase under the following conditions: 95 °C for 1 min, followed by 45 cycles of 95 °C for 30 s, 50 °C for 45 s, and a final extension at 72 °C for 5 min (PCR product 474 bp).

Nested PCR applications were performed using a similar reaction mixture (28 μL) with 3 μL of PCR products, 0.1 μM each forward primer (forward primer, 5′-CTTATTTGATGCGTCTACAGAG-3′; reverse primer, 5′-TTGACATGGGTGCACGTTACACATAC-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 2 min and a final extension at 72 °C for 5 min (PCR product 274 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 μM of each primer (2.21, P. vivax 2.21-PF 5′-GGCCAAGAGCTAGAGGAG-3′ and P. vivax 2.21-PR 5′-GGGTTGTTACATTTGGAGGTA-3′; 14.185, P. vivax 14.185-PF 5′-TGGCATATGGCTGTGCAAG-3′ and P. vivax 14.185-PR 5′-GGGAAACCCTGTTGTCAAC-3′; 8.332, P. vivax 8.332-PF 5′-TGAAGCAAATATAGCGATGAC-3′ and P. vivax 8.332-PR 5′-CGGTGTAGTGTGGTACAATG-3′; L40, P. vivax L40-PF 5′-ATTGTTGATGGCCTGTGTT-3′ and P. vivax L40-PR 5′-GTGAGGGTG-3′). The amplified products were run on a 3730xl DNA analyzer. Fragments were sized against a GeneScan 500 LIZ size standard on an ABI Prism 3730 XL DNA analyzer.


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

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<td>FY<em>B/FY</em>A^ES</td>
<td>Fya−/b+</td>
<td>0x Fya, 1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>B/FY</em>B^ES</td>
<td>Fya−/b+</td>
<td>0x Fya, 1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>X/FY</em>X</td>
<td>Fya−/b+^weak</td>
<td>0x Fya, 0.2x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>X/FY</em>A^ES</td>
<td>Fya−/b+^weak</td>
<td>0x Fya, 0.1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>X/FY</em>B^ES</td>
<td>Fya−/b+^weak</td>
<td>0x Fya, 0.1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>A/FY</em>B</td>
<td>Fya+/b+</td>
<td>1x Fya, 1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>A/FY</em>X</td>
<td>Fya+/b+</td>
<td>1x Fya, 0.1x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>A/FY</em>A^ES</td>
<td>Fya−/b−</td>
<td>0x Fya, 0x Fyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FY<em>A^ES/FY</em>B^ES</td>
<td>Fya−/b−</td>
<td>0x Fya, 0x Fyb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Duffy Phenotype</th>
<th>FY(a−b−)</th>
<th>FY(a+b−)</th>
<th>FY(a+b+)</th>
<th>FY(a−b+)</th>
<th>FY(a−b−):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duffy Genotype</td>
<td>FY*A/*A</td>
<td>FY<em>A</em>B⁵⁵</td>
<td>FY<em>A</em>B⁵</td>
<td>FY<em>B</em>B⁵</td>
<td>FY<em>B</em>B⁵⁵</td>
</tr>
<tr>
<td>Total population</td>
<td>25 (3.8%)</td>
<td>117 (17.7%)</td>
<td>11 (1.7%)</td>
<td>1 (0.1%)</td>
<td>31 (4.7%)</td>
</tr>
<tr>
<td><em>Pv</em> infection (prevalence)†</td>
<td>2 (8.0%)</td>
<td>33 (28.2%)</td>
<td>5 (45.5%)</td>
<td>—</td>
<td>4 (12.9%)</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection (prevalence)‡</td>
<td>12 (48.0%)</td>
<td>45 (38.5%)</td>
<td>6 (54.5%)</td>
<td>—</td>
<td>6 (19.4%)</td>
</tr>
<tr>
<td>Study site§</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andapa: Duffy genotype</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Farafangana: Duffy genotype</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Maevatanana: Duffy genotype</td>
<td>2</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mbindrivazo: Duffy genotype</td>
<td>8</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>9</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Moramanga: Duffy genotype</td>
<td>2</td>
<td>15</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tsiranoanomandidy: Duffy genotype</td>
<td>5</td>
<td>33</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>24</td>
<td>5</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>29</td>
<td>6</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Ejeda: Duffy genotype</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ihosy: Duffy genotype</td>
<td>4</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td><em>Pv</em> infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Plasmodium</em> sp. infection</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* 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†

<table>
<thead>
<tr>
<th>Duffy Phenotype</th>
<th>Fy(a+b−)</th>
<th>Fy(a+b+)</th>
<th>Fy(a−b+)</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient population</td>
<td>21 (11.5%)</td>
<td>95 (51.9%)</td>
<td>6 (3.3%)</td>
<td>7 (3.8%)</td>
</tr>
<tr>
<td>Pure Pv infection</td>
<td>20</td>
<td>84</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mixed Pv/Pf infection‡</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Study site§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maevatanana: Duffy genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Pv infection</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mixed Pv/Pf infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tsiraoanomandidy: Duffy genotype</td>
<td>12</td>
<td>49</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Pure Pv infection</td>
<td>11</td>
<td>38</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mixed Pv/Pf infection</td>
<td>7</td>
<td>29</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Moramanga: Duffy genotype</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pure Pv infection</td>
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<tr>
<td>Mixed Pv/Pf infection</td>
<td>—</td>
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<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ihosy: Duffy genotype</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pure Pv infection</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mixed Pv/Pf infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ejeda: Duffy genotype</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pure Pv infection</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Mixed Pv/Pf infection</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*A Duffy working nomenclature is reviewed in SI Appendix B. Only those Duffy genotypes/phenotypes associated with Plasmodium species infections during 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).