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*Bf*BF or BY) 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.


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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 SI], 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 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*. 52.2% 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* mono-infections. 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 individuals (Tsiraoanomandidy χ², 1 df = 2.87, *P* = 0.09; Miandrivazo χ², 1 df = 0.116, *P* = 0.733; Maevatanana χ², 1 df = 1.18, *P* = 0.278). In contrast to these village-specific findings, when considering all 661 school children surveyed, Duffy-negative individuals 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 school children were collected in 2006/2007 from febrile individuals seeking malaria treatment from health facilities (26). Among these, 153 carried *P. vivax* mono-infections 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* mono-infection 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* clinical 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. 2A and B) and *P. vivax* gametocytes (Fig. 2C-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 *FY*/*FY* antigemeric classifications. Fig. 3 illustrates flow cytometry phenotypes for the Duffy-positive and -negative Malagasy populations. One hundred eighty-three Duffy phenotypes were characterized as tropical (lightest gray), sub-desert (light gray), equatorial (middle gray), and highlands (dark gray). 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 control and the iso-type 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*BES/*BES [FY(a−b−)], 6 genotyped *FY*A/BES [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*BES/*BES 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*E₃ alleles and the *FY*B*E₃ GenBank.
zygosity (and VK247, genotyping of 16 isolates for PvCSP showed the presence of both 0.17 vs. 0.74 head-ache, and sweating without previous antimalarial treatment. Standard blood smear di-gnosis revealed a mixed infection with P. vivax divers-ity of Duffy gene.

Ménard et al. PNAS vol. 107 | no. 13 | March 30, 2010

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 develop-ment 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 con-sistent 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 find-ings, although the Duffy phenotype of the susceptible Liberian was not established.

Whereas new combinations of mutations or altered gene ex-pression 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^{3}/B^{5}) Malagasy. 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. 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^{3}/B^{5}), who presented at the Tsiranoamandidy 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 = 380 pRBCμL). 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 is characteristics of microgametocytes (male). D originated from a 12-year-old Duffy-negative (FY*B^{3}/B^{5}) male, who presented at the Mandrivazo 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 = 3,000 pRBCμL). 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^{3}/B^{5}) 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 pRBCμL). 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.
P. vivax readily infects erythrocytes of the squirrel monkey (Saimiri boliviensis) (29, 30). Whereas squirrel monkeys express an Fy-positive Duffy antigen (29–31), the P. vivax DBP binds poorly, if at all, to squirrel monkey erythrocytes (32), suggesting a PNDBP-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(A^E5) FY*f+a-) (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.

Materials and Methods

Populations and Conventional Parasite Diagnosis. Human subjects protocols (007/SANPF/2007 and 156/SANFPFS/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 Malagasy school 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-M; 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 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 (Aruscam) (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 Instage Matrix resin (BioRad) or directly from whole blood (100 μL) using proteinase K/phenol-chloroform.

Molecular Diagnosis. Molecular diagnosis evaluating SNP (Duffy −33, promoter −34, Codon 42, Fy*A vs. Fy*B) was performed using a post-PCR ligase detection reaction–fluorescence detection hybridization microplate assay (LDMA-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 PNDBP. 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 (Fya specific), HIro31 antibody (Fyb specific), and anti-Fy antibody (NaM185-2C3 clone, Duffy specific) (45). Briefly, erythrocytes from EDTA-anticoagulated fluid and control samples [Fy(a+b−)], Fy(a−b+), Fy(a+b+), and Fy(a−bweak) 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 IgGlG1M (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...
expected heterozygosity (H*) from haploid data and calculated as $H_* = [p(1−p)]$ [1 − ∑fi2] (fi is the number of isolates sampled, p* is the frequency of the H 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

Ménard et al. 10.1073/pnas.0912496107

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 at 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 mm) 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 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, Antafasy, and Zafiloro 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.

Tsiraoanomandy—latitude 18°77′S, longitude 46°04′E. Tsiraoanomandy 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 Amangan, 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 Maayn 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 was not observed in Barito River populations. In more recent studies by Hurles et al. four different Malagasy ethnic groups (Bezannozano, n = 6; Betseile, 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. Fy 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 five times through observation of associated Senegal, Benin, Bantu, Cameroon, and Arab-Indian haplotypes. In their 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 Oceanian 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\(^{E5}\) and FY*B\(^{B}\)). In the general survey inclusive of 661 individuals (1,322 alleles) the African FY*B\(^{E5}\) 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\(^{E5}\) 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 hemophilic (blood group name) who experienced a hemolytic transfusion reaction (11). The expected Fy\(^{a}\) antisera were discovered shortly thereafter in surveys of British populations; codominantly expressed Fy\(^{a}\) and Fy\(^{b}\) antisera 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\(^{a}\)” 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\(^{bE}\). 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-vectors promoter SNP was identified in a P-vectors promoter SNP was observed in the Malagasy epidemic region of Papua New Guinea (17); however, in Papua New Guinea this SNP is upstream from the ORF sequence that would otherwise encode Fy\(^{b}\), observed throughout Southeast Asia and Melanesia, allele designation FY*B\(^{bE}\).

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 → A) 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\(^{a}\) allele is consistently greater than that of Fy\(^{b}\), and this difference in frequency is much more pronounced in Papua New Guinea (23, 26); this is consistent with earlier surveys of Pacific Islanders with a high incidence of amino acid substitutions within the first intracellular loop of the Duffy protein and is associated with the Fy\(^{b}\) and Fy\(^{weak}\) 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\(^{weak}\) vs. Fy\(^{b}\) antigens when analyzed by flow cytometry (25); the allelic designation corresponding to the Fy\(^{weak}\) allele 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 Duffy alleles (FY*A, FY*B, FY*X, FY*A\(^{D5}\), and FY*B\(^{E5}\)) 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, \(\alpha\) (amino acid motif –CC–) and \(\beta\) (amino acid motif –CXCC–). 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

Ménard et al. www.pnas.org/cgi/content/short/0912496107 2 of 10
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 Fyα/Fyβ antigen site at codon 42 (ggt encodes G, Fyα; gct encodes D, Fyβ), and the Fyweak antigen site at codon 89 (gct encodes R, Fyα; tgc encodes C, Fyweak).

**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′-GGGAGGTAAAGCTTGCATG-3′; reverse primer, 5′-CTTCCGTGAACTCTGAAGTGG-3′; reverse primer, 5′-AGAGCTGAGGATACCCAG-3′) with 2.5 μM MgCl₂ 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′-GGGAGGTAAAGCTTGCATG-3′; reverse primer, 5′-AGAACACAGCAGGGAAATGAG-3′) and exon 2 codon region (forward primer, 5′-CTTCCGTGAACTCTGAAGTGG-3′; reverse primer, 5′-AGAGCTGAGGATACCCAG-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 MgCl₂, 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 forward and reverse primers, ABI PRISM BigDye terminator cycle sequencing ready reaction kit run on a 3730xl 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. S766830).

**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′-CAGGGCTTGGGCTGGG-3′; reverse primer, Duffy + 739dn 5′-CTGGCAGCTGAGGATACCCAG-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 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 mM each LDR probe, 1 μL of PCR product, and 2 units of Taq DNA ligase (New England BioLabs). LDR probes consisted of three 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:部

- **GTA-1** transcription factor binding site (−33 to +C): 5′-TTACACCATATATTTTTTTTAC-3′
- **C** tag 37: 5′-CTTTCATTTTCATTTTCATTCATAC-3′
- **ORF** tag 37: 5′-TTACACCATATATTTTTTTTAC-3′
- **ORF** tag 28: 5′-CTCAACAAACAAACACATTATCAACTCCAGATGACATAG-3′
- 5′-phosphate-tgcaacagtgaagcagtaa-biotin-3′.

Codon 42 encoding either the Fyα antigen or the Fyβ antigen: 5′-CAGGAATCCAAACACATTATCAACTCCAGATGACATAG-3′.

**Reaction mixtures.** Initial 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 consisting of 1 M tetramethylammonium chloride (TMAC), 50 mM Tris-HCl (pH 8.0), 3 mM EDTA (pH 8.0), 0.1% 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-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′-TACACCATATATTTTTTTTAC-3′; reverse primer, 5′-CAAACTCATATTCCATCATATTTTA-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 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′-GATGGGAGCTTTATATCCACCATC-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 3: 5′-tacctttcatcaatctactactcattctAGTTATATTAGTACT-3′

COI *P. falciparum* tag 5: 5′-aactttcatcatatcactacttCATTTGACAATGAT-3′

COI *P. ovale* tag 8: 5′-aactttcatcatatcactacttCATTTGACAATGAT-3′

COI *P. malariae* tag 62: 5′-aactttcatcatatcactacttCATTTGACAATGAT-3′

COI *P. species* common 1: 5′-phosphate-CTTTCAAGAAACTTTTGG-biotin-3′

COI *P. species* common 2: 5′-phosphate-CTTTCAAGAAACTTTTGG-biotin-3′

COI *P. species* common 3: 5′-phosphate-CTTTCACTGAT-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 aRotorGene 3000 thermocycler (Corbett Life Science); PCRs were performed in 25 μL reaction mixture containing 2.5 μL of sample DNA, 12.5 μL of qPCR MasterMix Plus for SYBR Green I No ROX (Eurogentec), and 0.6 μM of each primer (PL1473F18, 5′-aactttcatcatatcactacttCATTTGACAATGAT-3′; PL1679R18, 5′-TATCCAGA-biotin-3′). PCRs were performed using a similar reaction mixture (28 μL) containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-CGGATAGAGCACGAGCAG-3′; reverse primer, 5′-CCACCGTATGCTCT-3′), 3 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 mM 2-mercaptoethanol, 50 mM KCl, and 2.5 units of thermostable reaction mixture containing 3 μL DNA, 0.1 μM each primer (forward primer, 5′-TATCCAGA-biotin-3′; reverse primer, 5′-CCACCGTATGCTCT-3′), 3 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 mM 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. 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′-CGGATAGAGCACGAGCAG-3′; reverse primer, 5′-CCACCGTATGCTCT-3′), 3 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 mM 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′-etacaaatacaaatcataagGAGGATAGAG-CAGCTTGAGCACGACGCAGCAGCW-3′

ConservedVK210, 5′-phosphate-GGAATGGTTGAGAGT-GGACGGAGCAG-biotin-3′.

*P. vivax* subtype VK247 isolates: VK247tag80, 5′-ctaaataaatcataagGAGGATAGAG-CAGCTTGAGCACGACGCAGCAGCAGCW-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 was then 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′-AAATATACAGACACAAATTTCAT-3′; reverse primer, 5′-ATAAGAGTACGATACCTG-CG3′), 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 72 °C for 2 min 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′-CTATTATAGTGCTGCTAGAGAT-GAG-3′; reverse primer, 5′-TGACATGGTGATCCCTTACA-TAC-3′), under the following conditions: 95 °C for 1 min, followed by 45 cycles of 95 °C for 30 s, 50 °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 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 applications for the six microsatellite markers were done in a total volume of 20 μL that contained 0.1 μM of each primer (2.21, P2:21-PF 5′-GGCAGGAACCTAGAGAGGAG-3′ and P2:21-PR 5′-GGCTTGTCTTTATTTGGAGTA-3′; 14.185, P14:185-PF 5′-TGCAGATAGCTGCTGAAAT-3′ and P14:185-PR 5′-GGGAACATTTGGCTCAC-3′; 8.332, P8:332-PF 5′-TGAAGCAATATAGCGATGAC-3′ and P8:332-PR 5′-CGTGTTAGTTGTGTTACAATG-3′; L40, P4:40-PF 5′-ATTGTGATGTTGCTGTTT-3′ and P4:40-PR 5′-GTTAGGGTTCTATCCGTA-3′; 6.34, P6:34-PF 5′-CCCAATAATAGTCGACCAATCA-3′ and P6:34-PR 5′-CATGTAAAGAGGACCAT-GG-3′; and L34, P4:34-PF 5′-TACCCAGCTTTATCTCCT-3′ and P4:34-PR 5′-AAATGGAAGACACACTACG-3′), 200 μM of dNTPs, 2.5 mM of MgCl2, 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 μM of each specific primer labeled with a fluorescent dye (2.21, P2:21-NF 5′-6FAM-CCATCTGCTCAAATCGGAAG-3′ and P2:21-PR 5′-GGCACCCCTGGCTCTCTCC-3′; 14.185, P14:185-NF 5′-6FAM-GCAGTTGTCGACATGTCAG-3′ and P14:185-PR 5′-TAAAGGCGTGACCTATCAT-3′; 8.332, P8:332-NF 5′-HEX-CCCTGAGTTGATGTTGATG-3′ and P8:332-PR 5′-GTTAAAATCCGCCACCGACT-3′; L40, P4:40-NF 5′-HEXTACCAACGCCAATCTCC-3′ and P4:40-LR 5′-GTTC-ACCGGGGGTATACTAC-3′; 6.34, P6:34-NF 5′-6FAM-TGAGGCGTTTAAAGTTCTCG-3′ and P6:34-PR 5′-CAAAAATGAATCGTGGCACA-3′; and L34, P4:34-NF 5′-6FAM-TTCTCCCTGGAAAGAC-3′ and P4:34-LR 5′-ACGACCAT-CACCGTCCTAG-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), 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 3730xl 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.
Fig. S2. Peopling history of Madagascar and the Indian Ocean trade network (adapted from ref. 1).

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

<table>
<thead>
<tr>
<th>Allele†</th>
<th>Antigen</th>
<th>Genotype</th>
<th>Serological</th>
<th>Expression§</th>
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</thead>
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<td>Fy^a</td>
<td>FY<em>A/FY</em>A</td>
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<td>2x Fy^a, 0x Fy^b</td>
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<td>Fy^b</td>
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<td>1x Fy^a, 0x Fy^b</td>
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<td>FY<em>A/FY</em>B^ES</td>
<td>Fya+/b−</td>
<td>1x Fy^a, 0x Fy^b</td>
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</tr>
<tr>
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<td>Fy^b</td>
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<td>Fya−/b+</td>
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<td>0x Fy^a, 1x Fy^b</td>
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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>
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<th>Duffy Phenotype</th>
<th>FY(a+b−)</th>
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<th>FY(a−b+)</th>
<th>FY(a−b−):</th>
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</thead>
<tbody>
<tr>
<td>Duffy Genotype</td>
<td>FY*A/*A</td>
<td>FY<em>A</em>/B*ES</td>
<td>FY<em>B</em>B</td>
<td>FY<em>B</em>/B*ES</td>
</tr>
<tr>
<td>Total population</td>
<td>25 (3.8%)</td>
<td>117 (17.7%)</td>
<td>11 (1.7%)</td>
<td>31 (4.7%)</td>
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<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>
</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>
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Study site§

<table>
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<th>Location</th>
<th>Duffy genotype</th>
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<th>FY<em>A</em>/B*ES</th>
<th>FY<em>B</em>B</th>
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* 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.

Ménard et al. www.pnas.org/cgi/content/short/0912496107 9 of 10
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* 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).