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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sequences of the probes used were as follows:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sequences of the probes used were as follows:

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

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

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

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

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

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

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

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

1. Mouchet J, Blanchy S, Rakotonjanabelo A (1993) Epidemiological stratification of malaria in Madagascar. *Arch Inst Pasteur Madagascar* 60:50–59.
2. Julvez J (1995) [History of insular malaria in the southwestern Indian Ocean: An eco-epidemiologic approach]. *Sante* 5:353–358.
3. Burney DA, et al. (2004) A chronology for late prehistoric Madagascar. *J Hum Evol* 47: 25–63.
4. Dewar RE, Wright HT (1993) The culture history of Madagascar. *J World Prehist* 7: 417–466.
5. Fourquet R, Sarthou JL, Roux JF (1974) Sickle cell disease and Madagascar settlement, new hypothesis on its introduction in Africa. *Arch Inst Pasteur Madagascar* 43: 185–209.
6. Ottino P, Molet L (1972) Madagascar between Africa and Indonesia. *Homme* 12: 126–135.
7. Verin P (1986) *The History of Civilisation in North Madagascar* (AA Balkema, Rotterdam, Boston).
8. Hurles ME, Sykes BC, Jobling MA, Forster P (2005) The dual origin of the Malagasy in Island Southeast Asia and East Africa: Evidence from maternal and paternal lineages. *Am J Hum Genet* 76:894–901.
9. Soodyall H, Jenkins T, Stoneking M (1995) 'Polynesian' mtDNA in the Malagasy. *Nat Genet* 10:377–378.
10. Hewitt R, Krause A, Goldman A, Campbell G, Jenkins T (1996) Beta-globin haplotype analysis suggests that a major source of Malagasy ancestry is derived from Bantu-speaking Negroids. *Am J Hum Genet* 58:1303–1308.
11. Cutbush M, Mollison PL, Parkin DM (1950) A new human blood group. *Nature* 165: 188–189.
12. Ikin EW, Mourant AE, Pettenkofer HJ, Blumenthal G (1951) Discovery of the expected haemagglutinin, anti-Fyb. *Nature* 168:1077–1078.
13. Chaudhuri A, et al. (1993) Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the Plasmodium vivax malaria parasite. *Proc Natl Acad Sci USA* 90:10793–10797.
14. Tournamille C, Colin Y, Cartron JP, Le Van Kim C (1995) Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet* 10:224–228.
15. Iwamoto S, Li J, Sugimoto N, Okuda H, Kajii E (1996) Characterization of the Duffy gene promoter: Evidence for tissue-specific abolishment of expression in Fy(a-b-) of black individuals. *Biochem Biophys Res Commun* 222:852–859.
16. Woolley IJ, Hotmire KA, Sramkoski RM, Zimmerman PA, Kazura JW (2000) Differential expression of the duffy antigen receptor for chemokines according to RBC age and FY genotype. *Transfusion* 40:949–953.
17. Zimmerman PA, et al. (1999) Emergence of FY*A(null) in a Plasmodium vivax-endemic region of Papua New Guinea. *Proc Natl Acad Sci USA* 96:13973–13977.
18. Cavalli-Sforza LL, Menozzi P, Piazza A (1994) *The History and Geography of Human Genes* (Princeton Univ Press, Princeton).
19. Roychoudhury AK, Nei M (1988) *Human Polymorphic Genes: World Distribution* (Oxford Univ Press, New York).
20. Chaudhuri A, Polyakova J, Zbrzezna V, Pogo AO (1995) The coding sequence of Duffy blood group gene in humans and simians: Restriction fragment length polymorphism, antibody and malarial parasite specificities, and expression in nonerythroid tissues in Duffy-negative individuals. *Blood* 85:615–621.
21. Iwamoto S, Omi T, Kajii E, Ikemoto S (1995) Genomic organization of the glycoprotein D gene: Duffy blood group Fya/Fyb alloantigen system is associated with a polymorphism at the 44-amino acid residue. *Blood* 85:622–626.
22. Tournamille C, Le Van Kim C, Gane P, Cartron JP, Colin Y (1995) Molecular basis and PCR-DNA typing of the Fya/fyb blood group polymorphism. *Hum Genet* 95:407–410.
23. Olsson ML, et al. (1998) The Fy(x) phenotype is associated with a missense mutation in the Fy(b) allele predicting Arg89Cys in the Duffy glycoprotein. *Br J Haematol* 103: 1184–1191.
24. Parasol N, et al. (1998) A novel mutation in the coding sequence of the FY*B allele of the Duffy chemokine receptor gene is associated with an altered erythrocyte phenotype. *Blood* 92:2237–2243.

25. Tournamille C, et al. (1998) Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy(x) individuals. *Blood* 92:2147–2156.
26. Chown B, Lewis M, Kaita H (1965) The Duffy blood group system in Caucasians: Evidence for a new allele. *Am J Hum Genet* 17:384–389.
27. Murphy PM (1996) Chemokine receptors: Structure, function and role in microbial pathogenesis. *Cytokine Growth Factor Rev* 7:47–64.
28. Darbonne WC, et al. (1991) Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J Clin Invest* 88:1362–1369.
29. Fukuma N, et al. (2003) A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochem Biophys Res Commun* 303:137–139.
30. Nibbs R, Graham G, Rot A (2003) Chemokines on the move: Control by the chemokine "interceptors" Duffy blood group antigen and D6. *Semin Immunol* 15:287–294.
31. Pruenster M, et al. (2009) The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol* 10:101–108.
32. Carnevale EP, et al. (2007) A multiplex ligase detection reaction-fluorescent microsphere assay for simultaneous detection of single nucleotide polymorphisms associated with Plasmodium falciparum drug resistance. *J Clin Microbiol* 45:752–761.
33. McNamara DT, et al. (2006) Diagnosing infection levels of four human malaria parasite species by a polymerase chain reaction/ligase detection reaction fluorescent microsphere-based assay. *Am J Trop Med Hyg* 74:413–421.
34. Mehlotra RK, Ziats MN, Bockarie MJ, Zimmerman PA (2006) Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea. *Eur J Clin Pharmacol* 62:267–275.
35. de Monbrison F, Angei C, Staal A, Kaiser K, Picot S (2003) Simultaneous identification of the four human Plasmodium species and quantification of Plasmodium DNA load in human blood by real-time polymerase chain reaction. *Trans R Soc Trop Med Hyg* 97:387–390.
36. Singh B, et al. (1999) A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg* 60: 687–692.
37. Rosenberg R, et al. (1989) Circumsporozoite protein heterogeneity in the human malaria parasite Plasmodium vivax. *Science* 245:973–976.
38. Cole-Tobian JL, Zimmerman PA, King CL (2007) High-throughput identification of the predominant malaria parasite clone in complex blood stage infections using a multi-SNP molecular haplotyping assay. *Am J Trop Med Hyg* 76:12–19.
39. Barnadas C, et al. (2008) Plasmodium vivax dhfr and dhps mutations in isolates from Madagascar and therapeutic response to sulphadoxine-pyrimethamine. *Malar J* 7:35.
40. Imwong M, et al. (2006) Microsatellite variation, repeat array length, and population history of Plasmodium vivax. *Mol Biol Evol* 23:1016–1018.
41. Leclerc MC, et al. (2004) Meager genetic variability of the human malaria agent Plasmodium vivax. *Proc Natl Acad Sci USA* 101:14455–14460.

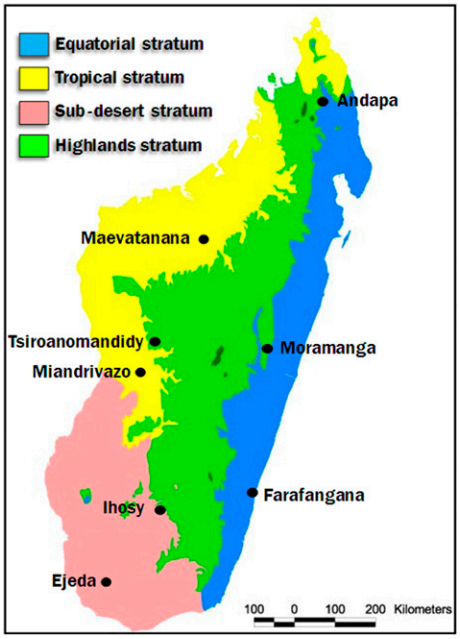

Fig. S1. Study sites and their distribution throughout Madagascar's four malaria ecological strata.

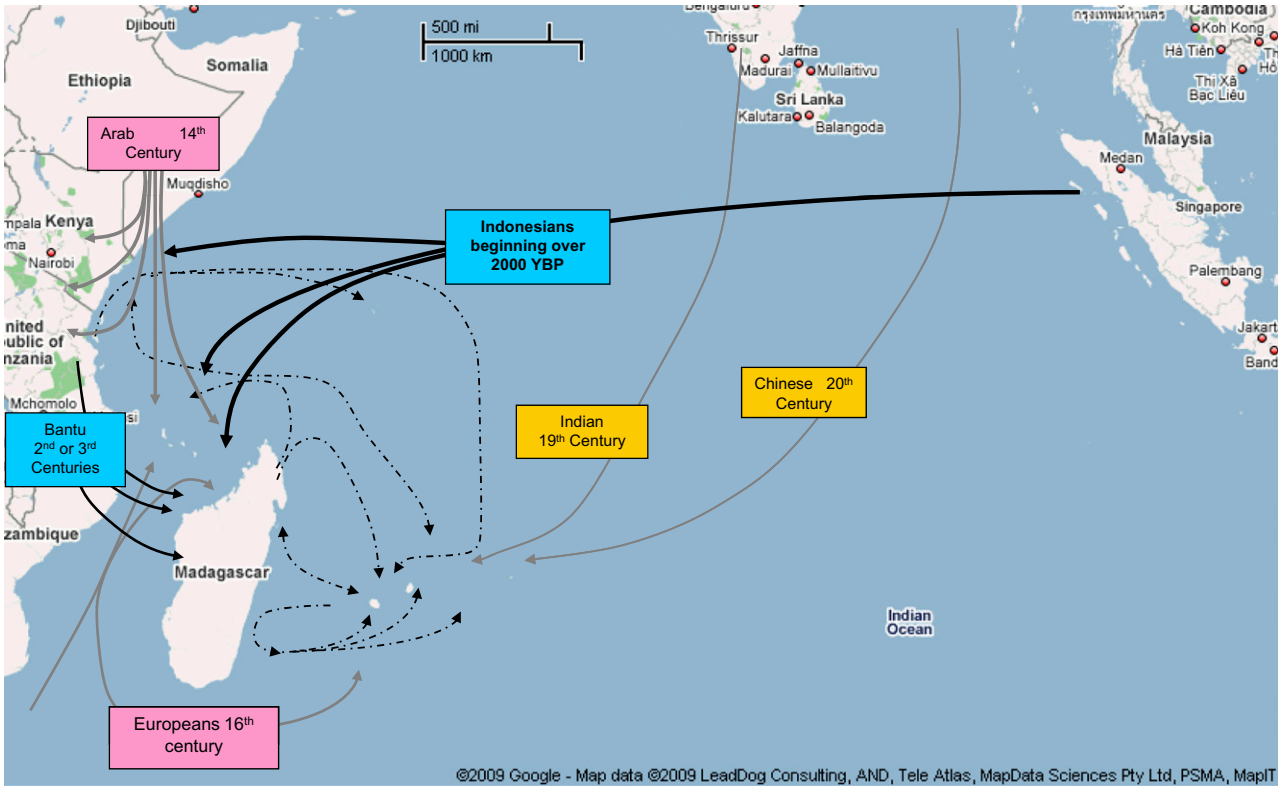

Fig. S2. Peopling history of Madagascar and the Indian Ocean trade network (adapted from ref. 1).

1. Julvez J (1995) [History of insular malaria in the southwestern Indian Ocean: An ecoepidemiologic approach]. *Sante* 5:353–358.

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

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

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

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

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

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

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

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

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

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

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

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