TABLE 4
Microsatellite haplotype diversity of *Plasmodium vivax* population in Turkey

Microsatellite locus	No.	Repeat unit	No. repeat types	Haplotype diversity (h)
MS8	30	CA(C/G)	3	0.476 ± 0.091
MS9	29	AGG	3	0.626 ± 0.048
MS15	29	CTT	3	0.488 ± 0.091
3.502	26	ATGAACGG	3	0.594 ± 0.073
				Mean ± SD = 0.546 ± 0.075

epidemiologic observations from areas highly endemic for *P. falciparum* malaria in sub-Sahara Africa, where a number of distinctive genotypes are circulating and mixed genotype infections are commonly seen.[46] Strain-specific protective immunity (SSPI) or allele-linked immunity is strongly suggested to be involved in the slow acquisition of protective immunity against *P. falciparum* malaria.[47] In a rodent malaria model, SSPI has been shown to exist,[48] and parasite MSP-1 has strongly been suggested to be the principal candidate molecule for the control of SSPI against *P. chabaudi chabaudi* malaria.[49,50] If strain-specific immunity is true for *P. vivax*, it is predicted that acquisition of immunity may be faster in Turkey than in other malaria-endemic areas, such as Thailand[13,32] and Brazil,[16] where *pvmsp1* diversity is relatively high. In Brazil, antibodies against polymorphic regions of PvMSP-1 are slow to develop, compared with the conserved C-terminal 19-kD polypeptide, which suggests that repeated infections are required to elicit antibody responses to variable sequence regions.[16] In Sri Lanka, antibody prevalence was higher to the 33-kD polymorphic region than to the 19-kD region.[51] Cohort studies to correlate protective immunity with antibody responses against PvMSP-1 (polymorphic regions and conserved regions, including the C-terminal 19-kD polypeptide) would be required to infer a role of *pvmsp1* polymorphism in immune evasion.

In general, the level of genetic diversity of the parasite may be determined by several variables such as population evolutionary/demographic history, effective population size, gene flow between neighboring populations, and natural selection. In a population that has an old origin and large effective population size, a higher genetic diversity would be expected than a population with a recent origin and small effective population size. Microsatellite diversity was lower in Turkey than in other areas, which suggested a relatively small effective population size in the parasite population studied. In Turkey, human movements from and to neighboring countries is strictly limited, and parasite gene flow accompanied by human movements would be too low to cause introduction of parasite variants not prevalent in Turkey or increase genetic diversity. The frequency distribution pattern of poly Q repeat haplotypes (Supplementary Figure 2) was distinctive between Turkey and neighboring countries (Azerbaijan and Iran), and the distribution pattern was apparently similar between Iran and Afghanistan. Contribution of human movements to increasing genetic diversity has been observed between Iran, Afghanistan and Pakistan.[23,30] In this study, Fu and Li's D* and F* tests showed significantly positive values for populations from Turkey, which suggested a recent parasite population bottleneck. Consistent with this finding is the dramatic decrease in annual incidence of malaria in Sanliurfa, where there were two waves of rapid reductions in the early 1980s and after 1999 until the present time (Figure 2).

Additionally, the present population genetic study indicates that a signature of balancing selection on *pvmsp1* appears differently among *P. vivax* populations. In Thailand, Brazil, and India, an excess of dN over dS was observed in the C-terminal 42-kD polypeptide; this finding was not detected in Turkey. The McDonald-Kreitman test detected balancing selection for the central and C-terminal polypeptides in Turkey, Thailand, and Brazil (and in India for the 42-kD fragment). A potential population bottleneck in the *P. vivax* populations in the study area after a rapid reduction of *P. vivax* cases caused by recent extensive malaria interventions may be associated with the failure of detecting an excess of dN over dS in Turkey.

In conclusion, the present study demonstrates low diversity of *pvmsp1* in *P. vivax* isolates from Turkey. The low antigen diversity should be informative for gaining insights of acquired immunity against *P. vivax* malaria. A population bottleneck of *P. vivax* was inferred, which was probably caused by recent malaria intervention efforts in the study area. A signature of balancing selection on *pvmsp1* was obscured in a parasite population that had experienced a bottleneck, which suggested geographic differences in balancing selection in an antigen gene of *P. vivax*.

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Report

Plasmodium falciparum Accompanied the Human Expansion out of Africa

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Summary

***Plasmodium falciparum* is distributed throughout the tropics and is responsible for an estimated 230 million cases of malaria every year, with a further 1.4 billion people at risk of infection [1–3]. Little is known about the genetic makeup of *P. falciparum* populations, despite variation in genetic diversity being a key factor in morbidity, mortality, and the success of malaria control initiatives. Here we analyze a worldwide sample of 519 *P. falciparum* isolates sequenced for two housekeeping genes (63 single nucleotide polymorphisms from around 5000 nucleotides per isolate). We observe a strong negative correlation between within-population genetic diversity and geographic distance from sub-Saharan Africa (R^2 = 0.95) over Africa, Asia, and Oceania. In contrast, regional variation in transmission intensity seems to have had a negligible impact on the distribution of genetic diversity. The striking geographic patterns of isolation by distance observed in *P. falciparum* mirror the ones previously documented in humans [4–7] and point to a joint sub-Saharan African origin between the parasite and its host. Age estimates for the expansion of *P. falciparum* further support that anatomically modern humans were infected prior to their exit out of Africa and carried the parasite along during their colonization of the world.**

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Results and Discussion

The genetic diversity of malaria parasites is central to their pathogenesis by facilitating immune evasion and drug resistance. As such, a better understanding of the worldwide distribution of the genetic diversity in *Plasmodium falciparum* is crucial for devising optimal drug- or vaccine-based malaria control strategies. Previous attempts at characterizing population structure in *P. falciparum* have pointed to considerable variation in genetic diversity and well-differentiated populations over the parasite's vast distribution range [8–14]. Moreover, no consensus has been reached over worldwide trends in the distribution of genetic diversity, with different studies identifying either Africa or South America as the region harboring the highest genetic diversity [8, 10, 13]. These inconsistencies between studies may be due to populations having been defined by pooling isolates sampled over heterogeneous geographic ranges. Alternatively, these discrepancies could be explained by the type of genetic markers that were deployed. Microsatellite markers may offer lower resolution for picking up patterns generated by relatively old demographic events as a result of their high mutation rate and high rate of back mutation (homoplasy). Conversely, single nucleotide polymorphisms (SNPs) often suffer from biases induced by the selection of polymorphic markers on a small initial discovery panel. This phenomenon, referred to as ascertainment bias, leads to the genetic diversity of populations that are not well represented in the initial discovery panel being strongly underestimated [15, 16].

The age of *P. falciparum* is also highly disputed [17–22], with previous estimates for the time to the most recent common ancestor (TMRCA) spanning more than an order of magnitude with values ranging from about 10,000 years [19, 22] to over 300,000 years [20]. With *P. falciparum* being an exclusively human parasite with no known animal reservoir, we hypothesized that if *P. falciparum* had been associated with humans for over 50,000 to 60,000 years (the estimated date for the out-of-Africa migration of anatomically modern humans), its current population structure could still carry a signal of human settlement history. Within-population genetic diversity of native human populations decreases smoothly with geographic distance measured through landmasses from a sub-Saharan African origin [5], and genetic differentiation between populations also increases steadily with physical distance along landmasses [7, 23]. These smooth patterns in the

distribution of human genetic diversity have been ascribed to sequential bottlenecks of small amplitude during the colonization of the world by our ancestors from an African cradle. In contrast, the parasite population structure may primarily depend on variation in epidemiological settings between populations. In particular, *P. falciparum* populations are characterized by high variability in variation in transmission intensity [1, 10, 18, 24, 25], which could have affected local genetic diversity. Selective pressure imposed by antimalaria interventions that used drugs and insecticides might, likewise, have locally reduced genetic diversity.

To assess the relative importance of past human demography and recent epidemiological factors, we used a data set of 519 *P. falciparum* isolates from nine populations covering the entire distribution range of the parasite, with populations from sub-Saharan Africa, Southeast Asia, Oceania, and South America. Care was taken to obtain a representative picture of the genetic makeup of *P. falciparum* populations by trying to minimize the geographic and temporal range of the isolates pooled into populations. We also chose to sequence two housekeeping genes, P type Ca^{2+}-ATPase (*serca*) and adenylosuccinate lyase (*adsl*), in their entirety for all isolates to circumvent any possible ascertainment bias induced by marker selection.

We identified 49 and 14 SNPs in the *serca* (3630 bp) and *adsl* (1413 bp) genes, respectively (see Table S1 available online). The ratio of synonymous (*dS*) over nonsynonymous (*dN*) substitutions did not significantly deviate from neutral expectations overall or in any of the nine parasite populations considered individually (Table S1). Because there was no evidence for natural selection, we present all results on analyses using both the synonymous and nonsynonymous SNPs. However, using synonymous SNPs only did not affect any of the conclusions (data not shown). Comparison of average pairwise nucleotide differences (θ_π) and standardized number of segregating sites (θ_S) indicates an excess of rare alleles in line with the high frequency of variants observed in a single population (private alleles), except in the two South American populations (Table S1; Figure S1).

Of the populations studied, those from Africa are by far the most genetically diverse, with the exception of Brazil, which is as diverse as Tanzania but only for the θ_π estimate for the *serca* gene (Table S1). However, we found no overall significant difference in within-population genetic diversity between the two genes. Thus, both genes were analyzed together in all subsequent analyses. The overall genetic differentiation is high, with a global F_{ST} = 0.21. Between-population genetic distances (pairwise F_{ST} estimates; Figure S2) are highest for the two South American populations, which are strongly differentiated from all other populations, with the largest of all pairwise differentiation observed between the two South American populations (F_{ST} = 0.47; Figure S2).

To test for an association between humans and *P. falciparum* predating the out-of-Africa exit, we computed the shortest distance through landmasses between each sampled population and a grid of hypothetical origins covering the entire world. We did not include the two South American populations in this analysis. The Americas were first colonized by humans some 15,000–20,000 years ago through the Bering Strait connecting Siberia to Alaska. The climate found today in the arctic is far too cold for the development of *P. falciparum*, which is unable to fulfill its life cycle at a temperature below 16°C–18°C [26, 27]. Because the temperature at the Bering Strait was considerably colder for the previous 90,000 years than it has been over the last 10,000 years [28], a joint colonization of *P. falciparum* together with humans into the Americas is highly implausible. Thus, the Americas have probably been colonized by this parasite far more recently, possibly through the slave trade [10, 17]. Interestingly, the resulting admixture could explain the relatively high level of parasite genetic diversity observed within American populations. We assumed that the cost of moving along coastlines was half the friction of movement inland. We also allowed for a southern route of migration out of Africa [29] by creating land bridges on either side of the Arabic Peninsula at the Bab-el-Mandeb Strait and Hormuz Strait.

Using these geographic distances and within-population genetic diversities (θ_π), we searched for the hypothetical origin providing the best correlation between genetic diversity and the logarithm of geographic distance (Figure 1). We observed the highest correlation for a central sub-Saharan African origin (R^2 = 0.95; p < 0.0001; Figure 1). Although this origin coincides with the one previously inferred for anatomically modern humans based on both genetic and morphological data [30], this result should be taken with some caution. The exact position of the inferred origin depends heavily on the genetic diversity of two African *P. falciparum* populations in the data set. Although the pattern is robust to the removal of either of the African populations (i.e., one at a time), ultimate confirmation of a coinciding geographic origin for both the parasite and its human host will require the inclusion of additional populations.

As previously reported in humans, there is also a strong isolation by distance (IBD) pattern, with pairwise genetic distances (pairwise F_{ST}) being tightly correlated with geographic distance, computed as above (Mantel r_M = 0.68; p < 0.001; Figure 2). The results are robust to the underlying assumptions in the analysis. We recover the same origin in sub-Saharan Africa with a correlation of R^2 = 0.95 if we use θ_S instead of θ_π genetic diversity estimates. Similarly, assuming an equal cost between coastlines and inland or a 3× lower cost for coastlines again points to a sub-Saharan African origin, with respective variance explained of 0.98 and 0.93 and IBDs of 0.46 and 0.66. Finally, forcing a northern route out of Africa through the Sinai into the Levant by closing the land bridges in and out of the Arabic Peninsula has a negligible effect on the best supported origin, with a correlation between geography and genetic diversity of R^2 = 0.95 and an IBD of r_M = 0.68.

The smooth patterns in the apportionment of *P. falciparum* genetic diversity mirroring the ones previously described in native human populations are suggestive of an extensive association between the parasite and its host. However, this is not sufficient to conclude that humans were infected prior to their migration out of Africa and carried the parasite along during their colonization of the Old World. *P. falciparum* genetic diversity may be driven by a variable that we did not consider but that is strongly correlated with distance from Africa. There are a variety of such candidate factors, ranging from the availability of competent insect vectors to resistance alleles in the human host. Because it is not feasible to consider sequentially a vast number of variables, we instead used the entomological inoculation rate (EIR), an inclusive metric capturing current transmission intensity, which has been previously shown to correlate with *P. falciparum* genetic diversity, as determined by microsatellite markers [10]. The intensity of malaria transmission varies greatly among geographic areas, with the highest transmission levels recorded in Africa and Oceania (particularly on Papua New Guinea and the

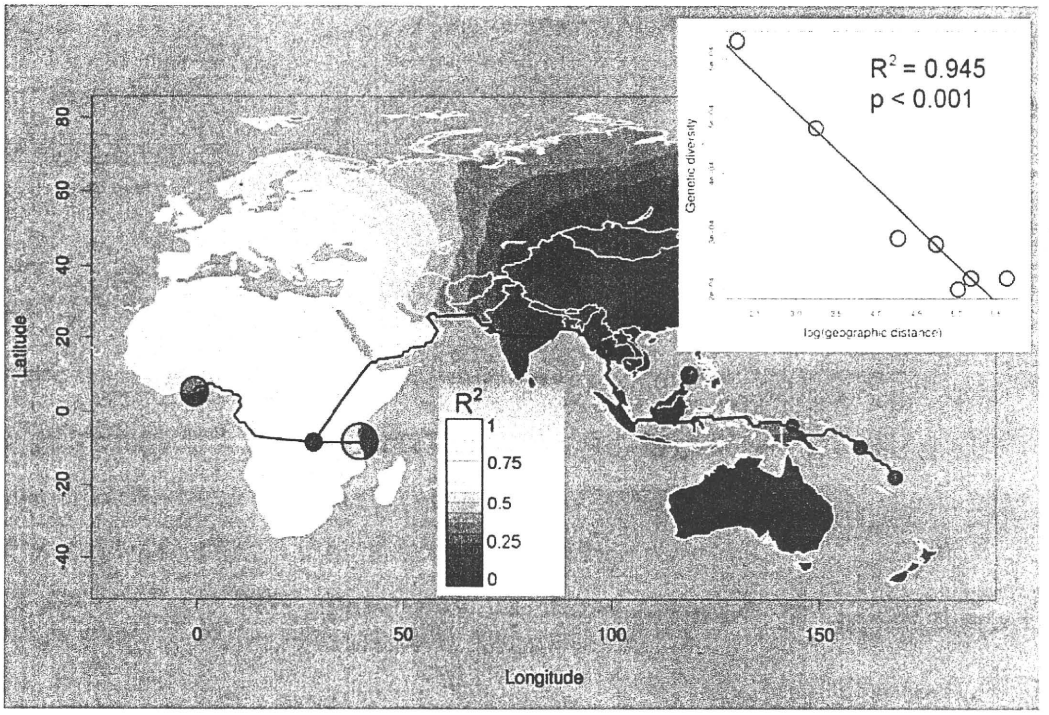

Figure 1. Map of Most Likely Origin for *Plasmodium falciparum*

Grey circles represent the geographic locations of the populations analyzed, with the surface of the circles proportional to within-population genetic diversity (θ_π). The background color represents the strength of the correlation between geographic distance from various origins and genetic diversity, with light shades representing more likely origins. The best supported origin is indicated by a blue dot. We also represented the colonization routes from this origin through landmasses to all populations analyzed (see Experimental Procedures). The inset represents the correlation between geographic distance (measured as travel cost over friction routes) and genetic diversity for the best supported origin.

Solomon Islands) and much lower transmission intensity in Southeast Asia (Thailand and the Philippines) [25, 31]. EIR rates were obtained for all non-American populations from the literature (Table S2). The correlation between distance from Africa and genetic diversity remains highly significant after controlling for EIR (R^2 = 0.81; p = 0.0015), suggesting that sequential bottlenecks during the expansion out of Africa are the main determinant of the global patterns in within-population diversity of *P. falciparum*.

Alternatively, *P. falciparum* may have originated in sub-Saharan Africa much more recently than anatomically modern humans but may have followed similar colonization routes to its human host during its expansion. To test for this possibility, we developed a new approximate Bayesian computation (ABC) framework of forward simulations of serial population expansions. This allowed us to estimate, among other parameters, the timing of the initial expansion of *P. falciparum*, as well as colonization rates and subsequent migration between colonized demes.

To parameterize the simulations, we needed to infer a substitution rate. To this end, we sequenced the two housekeeping genes in the chimpanzee malaria parasite *P. reichenowi* and estimated the substitution rate for the *serca* and *adsl* genes. There is no consensus on the exact timing of the split between *P. falciparum* and *P. reichenowi*. However, a range between around 2.5 million years ago (Mya) and 6 Mya is generally considered to be credible [21, 32–34]. There have also been speculations that the split between the two parasites may have coincided with the advent of agriculture in West Asia and could be as recent as 10,000 years ago [19, 22]. Although the latter calibration date leads to an implausibly high substitution rate for eukaryotic coding genes of about 10^{-6}/site/year, we still decided to consider this hypothesis of a very recent host transfer.

We ran 4 million simulations for each of the three substitution rates (based on the assumption of a 10,000 year, 2.5 Mya and 6 Mya split between the two malaria species). The 10,000 year split turned out to be highly implausible given the data, because we obtained not a single simulation with a reasonable fit to the observed pattern. Conversely, the model provided excellent fits between predicted and observed within-population genetic diversity $\ln(\theta_\pi)$, with R^2 = ~0.98 for the best combination of parameters for both scenarios with fast (split at 2.5 Mya) and slow (split at 6 Mya) mutation rates. The model with a faster mutation rate pointed to the start of the spread out of Africa at around 40 thousand years ago (Kya) (90% confidence interval: 19–77 Kya), whereas the slower mutation rate gave 80–90 Kya (33–96 Kya) (Figure 3; Table S3). These dates for the initial expansion of *P. falciparum* are compatible with the human out-of-Africa expansion some 50–60 Kya ago. Importantly, the 90% credibility intervals clearly exclude the advent of agriculture.

The demographic parameters were remarkably similar between the two scenarios (Figure S3). The only exception was the size of the ancestral population (K_0), which was greater for the slower mutation rate. This is the result of the TMRCA being accounted for by a combination of the starting time of the expansion and the TMRCA in the founding

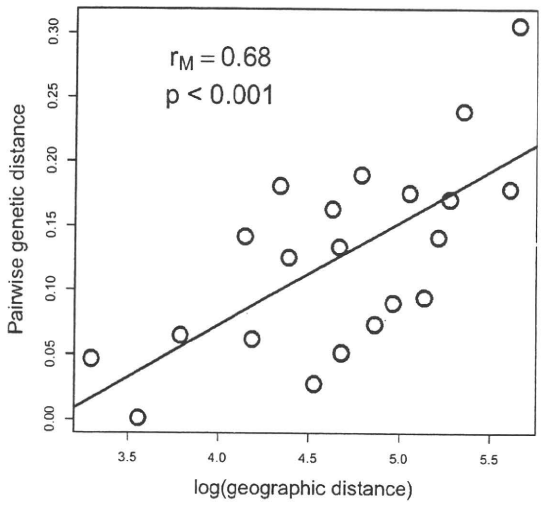

Figure 2. Plot of Isolation by Distance

Logarithm of pairwise geographic distances along landmasses (measured as travel costs over the friction routes) plotted against pairwise genetic distances (F_{ST}) between populations.

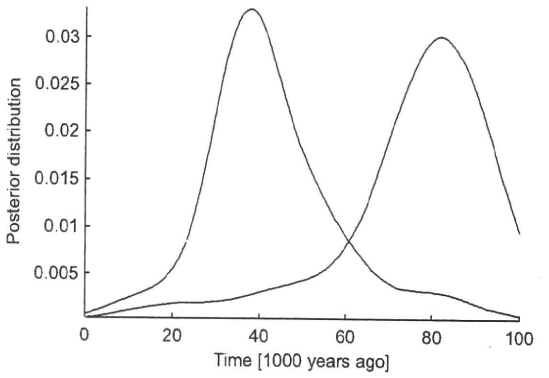

Figure 3. Posterior Distributions of the Date of Expansion of *P. falciparum*

These curves represent the posterior distributions of the date of expansion of *P. falciparum* obtained by the approximate Bayesian computation analysis. The two curves have been generated under the assumption of a split between the two *Plasmodium* species (*P. falciparum and P. reichenowi*) at 2.5 (blue) and 6 (red) million years ago.

population (determined by K_0). In general, both scenarios were characterized by a large ancestral population and relatively large carrying capacities for the demes during the expansion. Intense bottlenecks (Figure S3) were needed to achieve the sharp decrease in within-population genetic variability observed in the data. Population growth rate (which defines the speed of expansion) was relatively fast. Conversely, migration among demes was low, indicating that the pattern in genetic diversity was shaped primarily during the colonization of the tropics by *P. falciparum* rather than by subsequent exchanges between populations. Interestingly, the scenario we recover for the expansion of *P. falciparum* mirrors previous results for the human expansion out of Africa, with the same pattern of intense bottlenecks and low subsequent migration [35].

Our results establish that the genetic diversity of *P. falciparum* has been primarily shaped by human demography and does not provide evidence for a significant effect of contemporary nationwide malaria interventions based on mass drug administration and/or widespread spraying of DDT. For example, intensive malaria control programs in the Solomon Islands, initiated in the 1970s and since discontinued, led to a massive temporary reduction in parasite incidence [36]. Despite this, the genetic diversity found in the Solomon Islands does not fall below the curve for the genetic diversity, as predicted by distance from Africa (Figure 1). The results also suggest that geographic variation in the distribution of insect vector species may have played only a minor role in shaping the population structure of *P. falciparum*, despite extensive variation in anthropophily and behavior between Anopheles species [25].

In this paper, we have shown that the population genetic structure of *P. falciparum* outside of the Americas is primarily explained by geography, with 95% of the variance in within-population genetic diversity explained by physical distance from a sub-Saharan African origin alone. We further recovered strong patterns of isolation by distance and age estimates for the spread of *P. falciparum* coinciding with the colonization by anatomically modern humans of Africa, Asia, and Oceania. Taken together, our results confirm that *P. falciparum* had already infected humans before the out-of-Africa migration and followed our ancestors in their expansion throughout the tropics, with the exception of South America, which *P. falciparum* probably only reached much more recently through the slave trade. Although an association between humans and *P. falciparum* predating the exit out of Africa has been suggested before [18, 20, 37], our results additionally demonstrate the dramatic impact of past human migrations on the current apportionment of genetic diversity in the parasite.

Experimental Procedures

DNA Sample Collection

P. falciparum isolates were collected from nine countries: Tanzania, Ghana, Thailand, The Philippines, Papua New Guinea (PNG), the Republic of Vanuatu, the Solomon Islands, Brazil, and Venezuela. Details of the samples from Tanzania, Thailand, The Philippines, the Solomon Islands, Vanuatu, and Brazil used in this study have been described previously [31, 38–44]. Briefly, in Tanzania, blood samples were collected from infected individuals in the Rufiji River Delta in eastern coastal Tanzania in 1993, 1998, and 2003 [40]. In Thailand, blood samples were collected from malaria patients attending a malaria clinic in Mae Sot near the northwestern Thailand-Myanmar border in 1995 [42]. In the Philippines, samples were collected from malaria patients attending hospitals in Palawan Island in 1997 [31]. In the Solomon Islands, samples were collected from infected individuals in northeastern Guadalcanal Island in 1995–1996 [39]. In Vanuatu, samples were collected from four islands, Malakula, Gaua, Esprit Santo, and Pentecost, between 1996 and 1998 [38, 42]. In Brazil, isolates were collected from malaria patients in five states, Acre, Rondonia, Mato Grosso, Para, and Amapa, between 1985 and 1999 [43]. Additional samples were collected from Acre in 2004–2005 [44]. Venezuelan isolates were collected from Upper Orinoco, Amazonas State, Venezuela in 1997 (A.A.E., unpublished data). In all cases, ethical clearance for sampling was obtained from relevant ethical committees. Isolates from Ghana and PNG were collected specifically for this study. In Ghana, 182 *P. falciparum*-infected blood samples were collected during malaria surveys from 0- to 15-year-old children in three villages (Okyereko, Mpota, and Apam) near Winneba, a western coastal region, in November 2004. The study was approved by the Ministry of Health/Ghana Health Service. In PNG, 195 malaria-infected blood samples were collected during surveys in five villages in Wewak, East Sepik Province along the northeast coast: Kiniambu in August 2001, Jawia and Witupe in September 2001, and Boiken and Wingei in February 2002. The study was approved by the National Department of Health Medical Research Advisory Committee of PNG and the Tokyo Women's Medical University Ethical Committee. Informed consent was obtained from the patients or their

parents. In both Ghana and PNG, finger-prick blood was collected on Whatman 31ETCHR filter paper. Parasite genomic DNA was extracted from filter blots using the EZ1 DNA Investigator kit on the EZ1 BioRobot (QIAGEN).

DNA Sequencing

Isolates infected with mixed *msp1* haplotypes, as determined by polymerase chain reaction (PCR)-based haplotyping [31], were excluded from further analysis, and only those with a single *msp1* haplotype infection (n = 519) were used for sequencing. Full-length sequences were obtained for two housekeeping genes, Ca^{2+}-transporting ATPase gene (*serca*) and adenylosuccinate lyase gene (*adsl*). The Ca^{2+}-transporting ATPase of *P. falciparum* has recently been suggested to be a potential target of artemisinins [45], antimalarial drugs currently widely used for treatment in many endemic countries. However, all isolates in this study were collected before the adoption of this drug. Genomic DNA was subjected to two independent PCR amplifications, the products of which were directly sequenced in both directions. Procedures and conditions used for PCR amplification and sequencing of *serca* have been described elsewhere [46]. For amplification of *adsl*, the first PCR was run using primers ASL-F3 (5′-TATAACTCCCC AAAACAAAACCACTAAAATGT) and ASL-R4 (5′- AAAGGCGTACATGTTATA AGGTCCT), followed by a nested PCR using primers ASL-F2 (5′-ATTTATA TATATTCCTTATTATATAGTCA) and ASL-R3 (5′- TGGGAGTGCCCAACTTG CAGTGTCT). Full-length *adsl* sequence was also obtained from *P. reichenowi*, a chimpanzee malaria parasite closely related to *P. falciparum*. Whole-genome-amplified *P. reichenowi* genomic DNA [44] was subjected to PCR using three primer sets targeting the 5′, central, and 3′ regions of the gene: ASL-F3 and ASL-R6 (5′-CAATTATATAAGCATAAACCATATGCT), ASL-F6 (5′-AAATTGGAAGTAGTACCATGCCACA) and ASL-R4, and ASL-F5-2 (5′-GACAAATCATGATGTTAAGGCGGTTGA) and ASL-R5-2 (5′-CTTAT TGGTAATTTGGAACTAAATAACTTGA). PCR conditions were identical for *serca*. Whenever there was an inconsistency between two sequences after independent amplifications, a third round of PCR and sequencing was performed. Only isolates with a single genotype infection, as judged by the lack of overlapping peaks on electropherograms, were used for analysis. The sequences reported in this study have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database (accession numbers AB501575-AB502442, AB AB519183, and AB520081-AB520237).

Statistical Genetics Analyses

Two DNA sequence regions of Asn-codon tandem repeats in *serca* were excluded from the analyses. Nucleotide diversity was estimated by θ_π, the average pairwise nucleotide distance, and by θ_S, the standardized number of polymorphic sites per site (Watterson's estimator) using DnaSP version 4.10 [47]. A Z test was applied to test for a difference of in θ_S and θ_π between populations. The mean numbers of synonymous substitutions (*dS*) and nonsynonymous substitutions (*dN*) were estimated by the Nei and Gojobori method [48] with a Jukes and Cantor correction, as implemented in the MEGA software version 3.1 [49]. Standard error was determined by 1000 bootstrap replications, and *dN* and *dS* were compared with a Z test using MEGA. The interpopulation variance in allele frequencies, F_{ST}, was calculated using Weir and Cockerham's θ estimator [50] with Arlequin version 3.1 [51].

Spatially Explicit Analyses

All geographic distances were computed via graph theory [5, 23] as shortest distances along landmasses within a spherical referential of 40,962 vertices. We assumed that the friction cost along coastlines was half that of moving inland. Land bridges were created between the Malay Peninsula and Australia, connecting the major Indonesian islands. We further assumed two land bridges on either side of the Arabic peninsula at the Bab-el-Mandeb Strait and Hormuz Strait to allow for a southern route of colonization out of Africa [29]. The best supported origin was inferred by searching for the shortest routes to all analyzed populations from 312 hypothetical origins on land arranged on a regular grid. We controlled for EIRs using partial correlation. We tested whether within-population diversity was still significantly correlated to geographic distance from Africa once EIRs were accounted for first. For the isolation by distance analysis, a matrix of pairwise physical distances was computed for all populations using the distance through landmasses described above.

Approximate Bayesian Computation

We modeled the expansion of malaria out of Africa by considering a one-dimensional stepping stone, an approach successfully used to investigate the out-of-Africa expansion of anatomically modern humans, as well as *Helicobacter pylori* [6, 7, 35, 52]. Although the real expansion would have followed a two-dimensional spread, numerical studies suggest that a one-dimensional framework is a reasonable approximation as long as the distance between samples is large. We used 300 demes of equal sizes to represent the coastal route from the origin in Africa to Oceania (c.f. Figure 1). In order to avoid potential boundary effects at the origin, we added 50 demes to the African end (making the origin deme 51 in the chain).

Before the spread of *P. falciparum*, we imagine a well-mixed population of size K_o existed that represents the early presence of *P. falciparum* in Africa. At the onset of the spread, a seed population of size c_oK_o was placed at the origin. This population increased linearly with rate r until it reached size K, the carrying capacity for all demes in the simulation (in other words, K is the effective population size of a deme at carrying capacity). At each time step, demes that had reached their maximum size were allowed to send out colonists to adjacent empty demes and migrants to adjacent demes that had already been colonized. The number of colonists was given by the colonization rate c multiplied by the deme size K, and the number of migrants by the migration rate m multiplied by K (migrants were shared equally between the two adjacent neighbors). Once a deme was colonized by some individuals, its population increased linearly with growth rate r until it reached its maximum size, K. *P. falciparum* undergoes about six generations a year [10]. After testing that generation time (scaled for mutations) up to 60× slower (1/10 years) had no qualitative impact on the simulation results, we settled for a computationally reasonable compromise of one generation per year.

From the demography described above, we generated gene genealogies for the two unlinked genes (*serca* 3630 bp and *adsl* 1413 bp) according to the Wright-Fisher model: individuals were assumed to be randomly mating within each deme, and generations were nonoverlapping. Assuming a Poisson process, we then simulated mutations on the gene genealogies; three mutations rates, 9.18×10^{-7}/site/year, 3.67×10^{-9}/site/year, and 1.53×10^{-9}/site/year, were estimated from the data, assuming a divergence between *P. falciparum* and *P. reichenowi* at 10,000, 2.5 million, or 6 million years ago [19, 21, 22, 32–34]. For each simulated gene genealogy, we then computed for each deme θ_π the average number of pairwise differences per site between sequences within a deme. Rather than using the raw values of θ_π, which tend to have a highly skewed distribution, we applied a logarithmic transformation on θ_π, giving us a variable with an approximately symmetrical distribution.

We estimated the best parameter values that describe the spread of *P. falciparum* via ABC, including weighted local regression [53], using within-population $\ln(\theta_\pi)$ as our summary statistics. The stepping-stone parameter values for each simulation were sampled from uniform prior distributions of the log values of the following ranges: K 10–10^6, K_o 10–10^6, m 10^{-6}–0.5, c 10^{-6}–0.5, c_o 10^{-6}–1, and r 10^{-3}–1. The start of the simulation t had a uniform distribution from 1,000 to 100,000 years ago. We also enforced two constraints: $c\,K \geq 1$ and $c_o\,K_o \geq 1$. We ran 4 million simulations for each of the three mutation rates, with an acceptance criterion of $R^2 > 0.7$ between observed and predicted $\ln(\theta_\pi)$. For the fastest mutation rate (referring to a split between the *Plasmodium* species 10,000 years ago), we were unable to obtain any simulation that fitted the data well ($R^2 > 0.7$). For this reason, no results are shown, because this mutation rate is implausible given the data. For the other two mutation rates, we accepted 6352 and 8943 out of 4 million simulations for the split at 2.5 Mya and 6 Mya, respectively.

Accession Numbers

All sequences analyzed in this work have been deposited in the GenBank database under accession numbers AB501575-AB502442, AB519183, and AB520081-AB520237.

Supplemental Information

Supplemental Information includes three tables and three figures and can be found with this article online at doi:10.1016/j.cub.2010.05.053.

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Original Article

A Multiplex PCR-Based Molecular Identification of Five Morphologically Related, Medically Important Subgenus *Stegomyia* Mosquitoes from the Genus *Aedes* (Diptera: Culicidae) Found in the Ryukyu Archipelago, Japan

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SUMMARY: Internal transcribed spacer regions of ribosomal DNA were sequenced, and new species-specific primers were designed to simplify the molecular identification of five morphologically related subgenus *Stegomyia* mosquito species—*Aedes aegypti, Ae. albopictus, Ae. riversi, Ae. flavopictus*, and *Ae. daitensis*—found in the Ryukyu Archipelago, Japan. Each newly designed primer was able to amplify a species-specific fragment with a different size. Conditions for multiplex PCR were optimized to identify all five species in a single PCR. This method is a convenient tool for entomological field surveys, particularly in arbovirus endemic/epidemic areas where some of these species coexist.

INTRODUCTION

The subgenus of *Stegomyia* of the genus *Aedes* (Diptera: Culicidae) is a diverse species group as regards morphology and distribution (1,2). Indeed, many *Aedes* (*Stegomyia*) spp. have evolved as endemic species on isolated islands, especially in the South Pacific (2). Some mosquito species of this subgenus cause serious infectious diseases, such as dengue fever (DF)/dengue hemorrhagic fever (DHF), yellow fever, chikungunya fever, and other arbovirus-related diseases, in humans (3-5).

Five *Aedes* (*Stegomyia*) spp., namely *Aedes aegypti* (L.), *Aedes albopictus* (Skuse), *Aedes riversi* Bohart and Ingram, *Aedes flavopictus* Yamada, and *Aedes daitensis* Miyagi and Toma, have been described to date in the Ryukyu Archipelago, Japan, although *Ae. aegypti* has not been collected since the 1970s (6,7). The remaining four species continue to be collected in the Ryukyu Archipelago and/or mainland Japan, and their geographic distributions are known to overlap (6). However, although their larvae exhibit similar habitat requirements (6,7), the ecological niches of adult *Stegomyia* mosquitoes differ to some extent. Thus, whereas *Ae. aegypti*, the most domesticated species (3,4), and *Ae. albopictus* prefer vegetation in the vicinity of domestic environments, *Ae. riversi* and *Ae. flavopictus* are adapted to a forest environment (8). *Aedes galloisi* and *Aedes wadai*, which also belong to the subgenus *Stegomyia*, are rare species which are confined to the northern part of Japan, mountains of the Kyushu island and isolated islands (6).

Ae. aegypti and *Ae. albopictus* are distributed worldwide and are known to transmit dengue virus in Asia, the South Pacific, and the Americas. These species were first identified as dengue vectors of the epidemics in the early 20th century in the Ryukyu Archipelago and mainland Japan (3-5,9), although other mosquito species of this subgenus are also regarded as potential vectors in regions where they are found (10). As the DF/DHF vaccine is unavailable for practical use, transmission of the disease can only be prevented by reducing human-vector contact. The recent involvement of *Ae. aegypti* and *Ae. albopictus* in DF/DHF and chikungunya fever pandemics in many countries (11-16) has highlighted the need for correct identification of these mosquito species for vector control, as such vectors are expected to play different roles in transmission and often co-inhabit domestic environments (17).

Although *Stegomyia* spp. have similar white scale patterns, adult morphological characteristics, such as white scales on the scutum and white bands on the legs, are very useful in distinguishing the individual species (1). However, as larval characteristics overlap, damage to adult specimens during collection can often complicate the species identification process, therefore it is necessary to establish new identification methods that can be applied to morphologically damaged specimens. Owing to the importance of accurate identification, medical entomologists have recently paid attention to molecular techniques. Beebe et al. have developed a molecular identification technique for container-breeding mosquito species, including *Ae. aegypti* and *Ae.*

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albopictus in Australia, which allowed them to differentiate mosquito species using PCR (18). However, this method requires the use of restriction enzymes following PCR. In 2009, a combination of forward universal 5.8S and reverse primers specific to *Ae. albopictus* from Corsica was developed, although it was not subsequently confirmed that this primer was applicable to specimens from other regions (19).

In the present study, to simplify the molecular identification technique for *Stegomyia* spp., including the important dengue and chikungunya vectors, *Ae. aegypti* and *Ae. albopictus*, internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA), which can be used as genetic markers to differentiate mosquito species (20–22), were sequenced and new species-specific primers were designed. The PCR conditions were optimized for multiplex PCR. Furthermore, these primers were applied to species found in mainland Japan and other countries worldwide as well as the Ryukyu Archipelago. This new PCR method is expected to support rapid epidemiological surveillance to reduce the risks arising from introduction of viruses into Japan from neighboring countries, such as Taiwan and Southeast Asia.

MATERIALS AND METHODS

Mosquito samples: The females of five species of *Aedes*, subgenus *Stegomyia*, found in the Ryukyu Archipelago were used for the present study. As *Ae. aegypti* has not been collected in the last 30 years in either the Ryukyus or mainland Japan, a sample taken from a laboratory strain collected in Chiang Mai, Thailand was used. All samples collected in the field or obtained from laboratory strains were preserved in absolute ethanol prior to DNA extraction. *Ae. albopictus*, *Ae. flavopictus*, and *Ae. riversi* from mainland Japan were also included in the present analysis in order to apply the newly designed primers to specimens from all over Japan (Table 1). Generally, 1–3 female mosquitoes from each location were examined individually. The sequences available in GenBank were used for *Ae. flavopictus* (22). The taxonomy of the genus *Aedes* followed the Walter Reed Biosystematics Unit (WRBU) (23).

Cloning and sequencing: DNA extraction, PCR, electrophoresis, cloning, and sequencing were performed according to Toma et al. (21). Total DNA was extracted using the DNeasy tissue kit (Qiagen, Valencia, Calif., USA) and ITS regions of rDNA, including 18S, 5.8S, and 16S, were amplified by PCR using the forward primer: 18SFHIN, 5′-GTA AGC TTC CTT TGT ACA CAC CGC CCG T-3′, and the reverse primer: CP16, 5′-GCG GGT ACC ATG CTT AAA TTT AGG GGG TA-3′ (20,24). Each 50 μl reaction solution contained 30 ng of template DNA, 1× PCR buffer (Promega, Madison, Wis., USA), 0.1 mM each of dATP, dCTP, dGTP, and dTTP (Promega), 1.5 mM $MgCl_2$ (Promega), 2.5 U of Taq DNA polymerase (Promega), and 6 pmol of the primers described above. Amplification was performed using a program of 1 cycle at 97°C for 4 min, 30 cycles at 96°C for 30 s, 48°C for 30 s, 72°C for 2 min, and 1 cycle at 72°C for 4 min. Amplified PCR products (approximately 1,300 bp) were verified by 1.5% agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen). The purified fragments were cloned with pGEM-T Easy Vector (Promega) and *Escherichia coli* (Stratagene, La Jolla, Calif., USA). Two clones derived from a single female were processed in most experiments. All clones were purified using the QIAprep spin miniprep kit (Qiagen) and sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The sequencing reactions were extended in both directions using BigDye Terminator ver. 3.0 (Applied Biosystems) with forward primers T7, SP6, and CP17 complementary to 5.8S gene (24) and a reverse primer

Table 1. Origin of female mosquitoes examined

Mosquito species	Origin	Date of collection
Aedes (*Stegomyia*) *aegypti*	Chiang Mai, Thailand (F[1])	Laboratory strain (>F15)
Aedes (*Stegomyia*) *albopictus*	Akita Prefecture, Japan (F)	July, 2000
	Saitama Prefecture, Japan (F)	July, 2000
	Nagasaki Prefecture, Japan (F)	September, 2000
	Okinawa Prefecture, Japan (F)	June, 2000
	Ishigaki Island, Japan (F)	August, 2000
Aedes (*Stegomyia*) *flavopictus*[2]	Miyagi Prefecture, Japan	April to June, 1996
	Saga Prefecture, Japan	April to June, 1996
	Okinawa Prefecture, Japan	April to June, 1996
	Ishigaki Island, Japan	April to June, 1996
	Iriomote Island, Japan	April to June, 1996
Aedes (*Stegomyia*) *riversi*	Nagasaki Prefecture, Japan (F)	May, 2005
	Okinawa Prefecture, Japan (F)	January, 2003
	Ishigaki Island, Japan (F)	June, 2000
	Iriomote Island, Japan (F)	January, 2003
Aedes (*Stegomyia*) *daitensis*	Minami-Daito Island, Japan (F)	November, 2001

[1]: Fresh female samples collected in fields or obtained from laboratory strains were preserved in absolute ethanol prior to DNA extraction.
[2]: Data from GenBank (22).

Table 2. Newly designed reverse primers specific to five morphologically related *Aedes* (*Stegomyia*) mosquitoes in the Ryukyu Archipelago, Japan

Mosquito species	Reverse primer	Sequence of primer (5′ to 3′)
Aedes (*Stegomyia*) *aegypti*	aeg.r1	TAACGGACAC CGTTCTAGGC CCT
Aedes (*Stegomyia*) *albopictus*	alb.r1	GTACTAGGCT CACTGCCACT GA
Aedes (*Stegomyia*) *flavopictus*	fla.r3	ACCRCAAGCA AGCCTCRTCG TA
Aedes (*Stegomyia*) *riversi*	riv.r1	GTGTCGTCCG GGGTKAMCGT
Aedes (*Stegomyia*) *daitensis*	dai.r1	ACGGGTTGGT TGGCAAAAGC CGT

R = A/G; K = T/G; M = A/C.

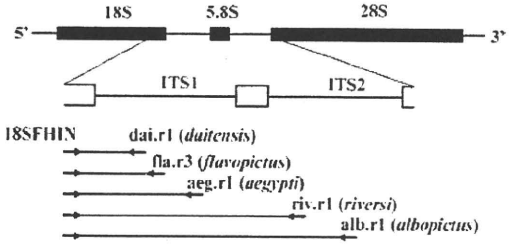

Fig. 1. rDNA gene group, the region amplified and sequenced, and the location and direction of primers used in the species identification assay.

58Sr complimentary to 5.8S gene (20).

Species-diagnostic PCR: The rDNA sequences were aligned using GENETYX ver. 7 (Genetyx, Tokyo, Japan) and Mega 3.1 software (Center for Evolutionary Medicine and Informatics, Phoenix, Ariz., USA). Variable sequence sites in the ITS1 and ITS2 regions for the five species were used to design species-diagnostic reverse primers with the forward primer 18SFHIN (Table 2, Fig. 1). The newly designed primers were examined individually to confirm that each one specifically amplified the expected size of fragments. To perform a species diagnosis in a single reaction, the PCR conditions were optimized as follows: 30 ng/μl template DNA; 1× PCR buffer (Promega); 0.04 mM each of dATP, dCTP, dGTP, and dTTP (Promega); 3.0 mM $MgCl_2$ (Promega); 1.0 U of Taq DNA polymerase (Promega); 2.4 pmol of each primer in 20 μl of reaction mixture; and 1 cycle at 96°C for 12 min, 40 cycles at 96°C for 30 s, 52°C for 30 s, 72°C for 90 s, and 1 cycle at 72°C for 4 min.

Identification of *Ae. aegypti* and *Ae. albopictus* from various countries: *Ae. aegypti* from Vietnam (Ho Chi Minh), El Salvador, Thailand (Chiang Mai), Indonesia (Bali) and Kenya (Kisumu), and *Ae. albopictus* from Japan (Nagasaki and Tokyo), Singapore, and Vietnam (Ho Chi Minh) were identified using our multiplex PCR method. For a region where *Ae. aegypti* and *Ae. albopictus* were expected to coexist, primers for these two species only were used for PCR identification.

Accession numbers for DNA sequences in GenBank database: The following accession numbers were used: *Ae. aegypti* (ITS1, AB548769-AB548774; ITS2, AB 548796-AB548801), *Ae. albopictus* (ITS1, AB548761-AB548768; ITS2, AB548788-AB548795), *Ae. riversi* (ITS1, AB548775-AB548782; ITS2, AB548802-AB548809), *Ae. flavopictus* (22); and *Ae. daitensis* (ITS1, AB548783-AB548787; ITS2, AB548810-AB548814).

RESULTS AND DISCUSSION

The primers designed on the basis of DNA sequences from ITS regions were found to amplify a specific fragment with a different size for each of the *Aedes* (*Stegomyia*) spp. inhabiting the Ryukyu Archipelago and mainland Japan. Fig. 2 shows that the primers used were able to effectively distinguish specific differences as different species had different bands, with the same band being visualized in the same species irrespective of its collection locality. Electrophoresis was performed for 1.5 h with 2.0% Nusieve agarose gel to differentiate *Ae. daitensis* from *Ae. flavopictus*. This process was necessary because of the only small difference in length of the PCR products for these two mosquito species (390 bp for *Ae. daitensis* and 410 bp for *Ae. flavopictus*; Table 3 and Fig. 2). This extensive electrophoresis may not, however, be a practical issue as *Ae. daitensis* is endemic to the Daito islands, which are isolated from the other regions inhabited by *Ae. flavopictus* (7). Although *Ae. daitensis* coexists with *Ae. albopictus* in the Daito islands, it is easy to distinguish one from the other on the basis of the much greater difference in length of their PCR products (Table 3, Fig. 2). Combinations of morphology, distribution records, and molecular information are useful for the identification

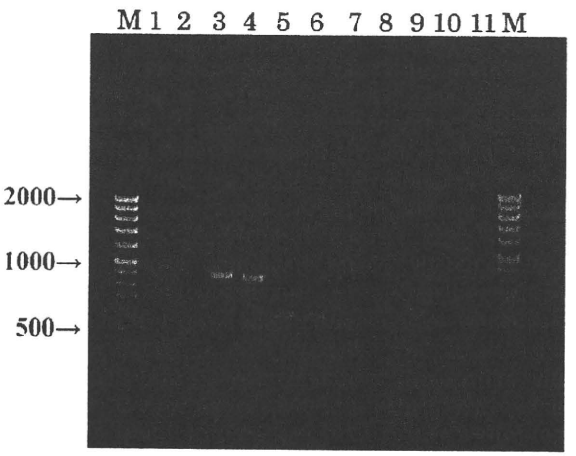

Fig. 2. Species-diagnostic PCR for five species of the subgenus *Stegomyia* in the Ryukyu Archipelago and mainland Japan. Lane 1, *Ae. albopictus* (Nagasaki); 2, *Ae. albopictus* (Okinawa); 3, *Ae. riversi* (Nagasaki); 4, *Ae. riversi* (Ishigaki Island); 5, *Ae. aegypti* (Thailand); 6, *Ae. aegypti* (Thailand); 7, *Ae. flavopictus* (Saga); 8, *Ae. flavopictus* (Okinawa); 9, *Ae. daitensis* (Minami-Daito Island); 10, *Ae. daitensis* (Minami-Daito Island); 11, Negative control. M, size marker. Females were used.

Table 3. The lengths of ITS1 and ITS2, and amplified sizes of PCR products for five morphologically related *Aedes* (*Stegomyia*) mosquitoes in the Ryukyu Archipelago, Japan

Mosquito species	Length of ITS1	Length of ITS2	Approximate amplified size[1)]
Aedes (*Stegomyia*) *aegypti*	425–435	206–217	550
Aedes (*Stegomyia*) *albopictus*	426–434	394–408	950
Aedes (*Stegomyia*) *flavopictus*	347–409	331–404	410
Aedes (*Stegomyia*) *riversi*	298–332	411–446	800
Aedes (*Stegomyia*) *daitensis*	328–330	384–401	390

[1)]: Including partial sequences of 18S (190 bp) for 5 species and 5.8S (155 bp) for *Ae. albopictus* and *Ae. riversi*.

of taxonomically related mosquito species.

Ae. flavopictus forms a morphologically diverse species complex (7), and genetic variations are also exhibited among the three subspecies of *Ae. flavopictus* in Japan (22). The species complex also exhibits differences in the length of the ITS regions in the present study. Thus, the variation in ITS length was larger in *Ae. flavopictus* (62 bp in ITS1, 73 bp in ITS2) than in *Ae. aegypti* (10 bp in ITS1, 11 bp in ITS2), *Ae. albopictus* (8 bp in ITS1, 14 bp in ITS2), *Ae. riversi* (34 bp in ITS1, 35 bp in ITS2), and *Ae. daitensis* (2 bp in ITS1, 17 bp in ITS2) (Table 3). As the conserved sequences in ITS1 were chosen for a reverse primer, an equivalent size of PCR products was obtained for the *Ae. flavopictus* spp. complex (Fig. 2).

It was worth demonstrating that the difference in length of the ITS region among *Ae. riversi* populations is relatively large despite the minimal difference suggested by electrophoresis (Table 3, Fig. 2). *Ae. riversi* is common and widely distributed among the islands of the Ryukyu Archipelago and the Kyushu and Shikoku islands, although its known habitats in the latter islands are confined to coastal areas in lowland-type natural forests of evergreen broad-leaved trees (lowland-type lucidophyllous forests) (6,7,25,26). In light of its unique distribution in the Kyushu and Shikoku islands, two hypotheses were suggested to explain the present distribution of this species. One of these hypotheses proposed that the species could expand as pioneers originating from the Ryukyu Archipelago, whereas the other proposed that this species had distributed continuously when the Kyushu islands formed part of the Asian continent (26). The common occurrence of *Ae. riversi* on several continental islands ranging from Danjo to Tsushima in Nagasaki, Kyushu strongly supports the latter hypothesis and suggests that the present distribution is a relic remaining after environmental changes on the Kyushu and Shikoku islands depleted the original populations (26). The lengths of ITS1 and ITS2 were 298–320 bp and 411–426 bp, respectively, for the population from the Ryukyu Archipelago population, and 328–332 and 413–446 bp, respectively, for specimens from the Kyushu island population (Nagasaki). Although there was no reproductive isolation between the Ryukyu Archipelago and Kyusyu island populations (27), the fact that the ITS is longer in *Ae. riversi* in Kyushu than in the Ryukyu Archipelago suggests some habitat-related speciation of this species. Further study is, however, required to determine the geographical origin of this species.

The distribution of two major dengue vectors, *Ae. aegypti* and *Ae. albopictus*, has changed dramatically over the last two decades. *Ae. albopictus* has expanded into new regions outside its original habitat (3,28), and the relative abundance of *Ae. aegypti* and *Ae. albopictus* has varied in many regions due to environmental changes resulting from human activities that favor either *Ae. aegypti* or *Ae. albopictus* (3). Dynamic differences in the infestation of both species are therefore likely to influence the epidemiological features of DF/DHF or other arbovirus-linked diseases in these regions, thus meaning that the accurate identification of dengue vectors is critical. The newly designed primers reported herein were found to amplify a specific DNA fragment with a different size for each species from various countries (Fig. 3). These species-specific primers can therefore be used to identify specimens, especially those from dengue and chikungunya epidemic/endemic areas, where *Ae. aegypti* and *Ae. albopictus* coexist. At the same time, they should be useful for rapid surveillance for a new introduction of either *Ae. aegypti* or *Ae. albopictus*. In such an event, it would be relatively straightforward to collect the eggs of the suspected invader, identify the species and link the result to an appropriate control program. Indeed, we have confirmed the utility of the species-specific fragments described in this study using the DNA from a single egg (Higa et al., unpublished).

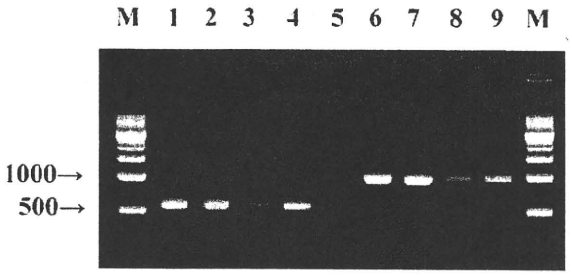

Fig. 3. Species-diagnostic PCR for *Ae. aegypti* and *Ae. albopictus* from various countries. Lanes 1–5, *Ae. aegypti*; 1, Vietnam (Larva, F6); 2, El Salvador (Larva, F2); 3, Thailand (Female, >F50); 4, Indonesia (Female, 2008 field collected); 5, Kenya (Larva, 2009 field collected). Lanes 6–9, *Ae. albopictus*; 6, Singapore (Larva, F3); 7, Nagasaki, Japan (Larva, >30F); 8, Tokyo, Japan (Female, 2005 field collected); 9, Vietnam (Larva, F12). M, size marker. 2% agarose gel was used.

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平成２２年度業績

＊研究成果の刊行に関する一覧表

＊学会発表一覧表

研究成果の刊行に関する一覧表 (平成２２年度)

執筆者氏名	刊行書籍又は雑誌名 (雑誌のときは雑誌名、 巻号数、論文名)	刊行書店名	巻名	ページ	刊行年
プロジェクト１：細菌					
Izumiya H, Pei Y, Terajima J, Ohnishi M, Hayashi T, Iyoda S, Watanabe H.	New system for multilocus variable-number tandem-repeat analysis of the enterohemorrhagic Escherichia coli strains belonging to three major serogroups: O157, O26, and O111.	Microbiol Immunol	54	569-577	2010
Morita M., Ohnishi M., Arakawa E., Yamamoto S., Nair G.B., Matsushita S., Yokoyama K., Kai A., Seto K., Watanabe H., Izumiya H	Emergence and genetic diversity of El Tor *Vibrio cholerae* O1 that possess classical biotype *ctxB* among travel-associated cases of cholera in Japan.	J. Med. Microbiol	59	708-712	2010
Mitobe J, Morita-Ishihara T, Ishihama A, Watanabe H.	2009 Involvement of RNA-binding protein Hfq in the osmotic-response regulation of *invE* gene expression in *Shigella sonnei*.	BMC Microbiol	9	110	2009
Teh CS, Thong KL, Osawa R, Chua, KH	Comparative PCR-based fingerprinting of Malaysian *Vibrio cholerae*.	J Gen Appl Microbiol		(in press)	
プロジェクト２：ウイルス（デング熱）					
Ito, M., Mukai, RZ., Takasaki, T., Kotaki, A. and Kurane, I.	Antibody-dependent enhancement of dengue virus infection in vitro by undiluted sera from monkeys infected with heterotypic dengue virus.	Archives of Virology.	155(10)	1617-1624	2010
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Moi, M.L., Takasaki, T., Kotaki, A., Tajima, S., Lim, C.K., Sakamoto, M., Iwagoe, H., Kobayashi, K., and Kurane I	Importation of dengue virus type 3 to Japan from Tanzania and Cote d'Ivoire.	*Emerging Infectious Diseases*	16	1770-1772	2010
Tajima, S., Takasaki, T., and Kurane, I.	Restoration of replication-defective dengue type 1 virus bearing mutations in the N-terminal cytoplasmic portion of NS4A by the additional mutations in the NS4B.	*Archives of Virology*	156	63-69	2011
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C.K., Kurane, I., Kase, T., Takahashi, K.	Antibodies over a 6-Month Period.				
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プロジェクト 3：ウイルス（高病原体 H5N1 鳥インフルエンザ）

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Yuma Iwai, Hitoshi Takahashi, Dai Hatakeyama, Kazunori Motoshima, Minoru Ishikawa, Kazuyuki Sugita, Yuichi Hashimoto, Yuichi Harada, Shigeyuki Itamura, Takato Odagiri, Masato Tashiro, Yoshihisa Sei, Kentaro Yamaguchi, Takashi Kuzuhara.	Anti-influenza activity of phenethylphenylphthalimide analogs derived from thalidomide.	Bioorg Med Chem	18	5379-90	2010