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# *Plasmodium falciparum* mitochondrial genetic diversity exhibits isolation-by-distance patterns supporting a sub-Saharan African origin ☆

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ABSTRACT

The geographical distribution of single nucleotide polymorphism (SNP) in the mitochondrial genome of the human malaria parasite *Plasmodium falciparum* was investigated. We identified 88 SNPs in 516 isolates from seven parasite populations in Africa, Southeast Asia and Oceania. Analysis of the SNPs postulated a sub-Saharan African origin and recovered a strong negative correlation between within-population SNP diversity and geographic distance from the putative African origin over Southeast Asia and Oceania. These results are consistent with those previously obtained for nuclear genome-encoded housekeeping genes, indicating that the pattern of inheritance does not substantially affect the geographical distribution of SNPs.

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## 1. Introduction

Malaria, a mosquito-borne infectious disease caused by protozoa of the genus *Plasmodium*, continues to impose a serious public health concern throughout the tropics with 219 million estimated cases and 660,000 deaths in 2010 (WHO, 2012), mostly due to infections of the most virulent species, *Plasmodium falciparum*. Elucidating and understanding the genetic diversity of this parasite is of great importance to gain insights into its pathogenesis as this has compromised malaria control strategies by facilitating drug resistance and immune evasion.

Malaria parasite populations in geographically diverse regions exist in highly variable epidemiological settings with respect to intensity of transmission (Hay et al., 2009; Kelly-Hope and McKenzie, 2009), malaria interventions, evolutionary history and population structures (Anderson et al., 2000; Joy et al., 2003). We have recently shown that within-population diversity of *P. falciparum* housekeeping genes was primarily determined by geographic distance from a postulated origin in central sub-Saharan Africa over Africa, Asia and Oceania (Tanabe et al., 2010). In a subsequent study, we also recovered significant negative (or inverse) correlation between within-population diversity of *P. falciparum* vaccine candidate antigen genes and geographic distance (Tanabe et al., 2013). These results suggest that regional variation in transmission intensity, malaria interventions, and host immune level might not be tightly associated with the geographic distribution of within-population parasite

*Abbreviations:* mt, mitochondria; SNP, single nucleotide polymorphism; $\theta_S$, the standardized number of polymorphic sites per site; $\theta_\pi$, the average number of pair-wise nucleotide differences; dS, the mean number of synonymous substitutions per synonymous site; dN, the mean number of non-synonymous substitutions per non-synonymous site; $F_{ST}$, the Wright's fixation index of inter-population variance in allele frequencies; MAF, minor allele frequency; *serca*, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-transporting ATPase gene; *adsl*, adenylosuccinate lyase gene; ATQ, atovaquone; *cox1*, cytochrome *c* oxidase subunit I gene; *cox3*, cytochrome *c* oxidase subunit III gene; *cob*, cytochrome *b* gene.

☆ We dedicate this paper to the memory of the late Kazuyuki Tanabe.

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genetic diversity. Rather, the smooth patterns of reduction in parasite genetic diversity along with geographic distance from the African origin mirror the ones previously documented in modern humans (Handley et al., 2007; Liu et al., 2006; Prugnolle et al., 2005; Ramachandran et al., 2005). We then inferred that *P. falciparum* "piggybacked" with modern humans in a central sub-Saharan area and colonized the world alongside modern human migrations out of Africa (Tanabe et al., 2010).

Like other eukaryotic organisms, *P. falciparum* has mitochondria essential for a range of cellular processes and cellular signaling. The parasite mitochondria showed remarkable changes in both morphology and biochemical activities during parasite development (Krungkrai, 2004; Vaidya and Mather, 2009). In erythrocytic stages, although the organelle has underdeveloped structure and relatively limited function, enzymes for oxidative phosphorylation were expressed, suggesting heterogeneity of metabolic status at the blood stage (Daily et al., 2007). At the mosquito stages of the parasite, the importance of TCA cycle members has recently been demonstrated (Hino et al., 2012) consistent with the organelle being metabolically more active and developed showing typical cristate morphology.

Additionally the mitochondrial (mt) genome of *P. falciparum*, as well as other *Plasmodium* species, has several unique genomic features (Vaidya and Mather, 2009): (i) the genome is present in the form of a circular and/or tandemly repeated linear 6 kb element, the smallest mt genome size known so far (Preiser et al., 1996); (ii) the 6 kb element encodes only three protein-coding genes (*cox1*, *cox3* and *cob*: cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit III and cytochrome *b* genes, respectively) along with highly fragmented large subunit (LSU) and small subunit (SSU) ribosomal RNA (rRNA) genes (Feagin et al., 1997, 2012; Hikosaka et al., 2011); and (iii) curiously, no transfer RNA genes exist.

These unique genomic features may impact strongly the genetic diversity observed in parasite populations. Moreover, similar to a great majority of sexual organisms, the parasite mt genome is uniparentally inherited and does not undergo recombination (Creasey et al., 1993; Joy et al., 2003; Vaidya et al., 1993). Thus, it is likely possible that the geographic distribution of within-population genetic diversity of the mt genome is different from that of the nuclear genome, which is inherited biparentally and undergoes recombination. For example, once a deleterious mutation is newly generated in the mt genome, all single nucleotide polymorphisms (SNPs) previously present in the genome must disappear, due to the absence of recombination in the mt genome. In contrast, in the nuclear genome only SNPs linked to a newly generated deleterious mutation in a chromosomal recombination segment would be lost by recombination and genetic hitchhiking with SNPs outside the segment surviving. This may suggest that geographical distribution of SNPs in the mt genome could be inherently unique. However, investigations on polymorphisms of the *P. falciparum* mt genome are still limited (Conway et al., 2000; Joy et al., 2003). There is also a clinical and epidemiological reason for studying polymorphisms of the mt genome relating to its biological role. Atovaquone (ATQ), an anti-malarial drug, is known to target cytochrome *b*, leading to the inhibition of pyrimidine biosynthesis and eventually parasite death (Korsinczky et al., 2000; Srivastava et al., 1999). The prevalence of ATQ-resistant parasites in targeted populations needs to be assessed before and after implementation of the drug usage for effective treatment and malaria control.

In this study, we investigated the geographical distribution of SNPs in the *P. falciparum* mt genome with the same parasite populations from Africa, Southeast Asia and Oceania that were previously used for the analysis of nuclear genome-encoded housekeeping genes (Tanabe et al., 2010). Using the same sample set allows us to address whether within-population mt SNP diversity is driven by a negative correlation with the geographic distance from a sub-Saharan African origin, as in nuclear genome-encoded housekeeping genes (Tanabe et al., 2010), or whether specific genetic features such as uniparental inheritance and the absence of recombination in the mt genome determine the geographic distribution of mt SNPs.

## 2. Materials and methods

### 2.1. Parasite isolates

*P. falciparum* isolates were collected from seven countries: Ghana, Tanzania, Thailand, Philippines, Papua New Guinea (PNG), the Solomon Islands, and Vanuatu. Details of the parasite isolates have been described previously: in Ghana, samples were collected in three villages near Winneba, coastal Ghana in 2004 (Tanabe et al., 2010). In Tanzania, samples were collected in the Rufiji River Delta in 1993, 1998 and 2003 (Tanabe et al., 2007). In Thailand, samples were collected in Mae Sod, Tak Province in 1995 (Sakihama et al., 1999). In the Philippines, samples were collected in Puerto Princesa, Palawan island in 1997 (Sakihama et al., 2007). In Papua New Guinea (PNG), samples were collected from five villages in Wewak, East Sepik Province in 2001 and 2002 (Tanabe et al., 2010). In the Solomon Islands, samples were collected in three local areas in northeastern Guadalcanal island in 1995–1996 (Sakihama et al., 2006). In Vanuatu, samples were collected from four islands in 1996–1998 (Sakihama et al., 2001). These seven parasite populations were also used in our previous polymorphism study for two nuclear housekeeping genes, the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-transporting ATPase gene (*serca*) and the adenylosuccinate lyase gene (*adsl*) (Tanabe et al., 2010). Ethical approval was obtained from relevant ethical committees.

### 2.2. Sequencing

Near full-length sequences (5883–5885 bp) of the *P. falciparum* mitochondrial genome were amplified by PCR using Takara LA Taq (Takara Bio, Japan). Procedures and conditions for PCR amplification were described (Tanabe et al., 2011). Four sets of primers were used in two successive reactions. The 5′ 3-kb region was first amplified using primers mtF0 and mtR3076, followed by a second PCR using primers mtF1 and mtR3076; the 3′ 3-kb region was first amplified using primers mtF2992 and mtR1, followed by a second PCR using primers mtF3019 and mtR2 (Supplementary Table 1). The PCR products were purified using QIAquick (PCR Purification Kit, QIAGEN). DNA sequencing was performed directly from two independent PCR products, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequencing primers were designed to cover target regions in both directions (Supplementary Table 1). Mixed genotype infections, judged from overlapping peaks at given positions in an electropherogram, were excluded from further analysis. We obtained 516 sequences from the seven parasite populations. The nucleotide sequences were deposited in the DDBJ/EMBL/GenBank database (accession numbers: AB570434–AB570542, AB570544-AB570765, AB570767-AB570951).

### 2.3. Statistical analysis

DNA sequences were aligned as described in Tanabe et al. (2011), and insertions/deletions (indel), were excluded. The aligned sequences (5883 bp) were subjected to statistical analysis and the following statistical indices were calculated: number of haplotypes, number of polymorphic sites, number of singleton polymorphic sites, the average number of pair-wise nucleotide differences per site ($\theta_{\pi}$), and the standardized number of polymorphic sites per site ($\theta_S$), i.e., Watterson's estimator. All statistics were calculated by DnaSP version 5.00 (Librado and Rozas, 2009). The mean numbers of synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) were estimated using MEGA 5.0 (Tamura et al., 2011). When dN was significantly higher than dS, diversifying

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