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Research paper

Genetic diversity and population structure of *Plasmodium falciparum* over space and time in an African archipelago

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ABSTRACT

The archipelago of São Tomé and Principe (STP), West Africa, has suffered the heavy burden of malaria since the 16th century. Until the last decade, when after a successful control program STP has become a low transmission country and one of the few nations with decreases of more than 90% in malaria admission and death rates.
We carried out a longitudinal study to determine the genetic structure of STP parasite populations over time and space. Twelve microsatellite loci were genotyped in *Plasmodium falciparum* samples from two islands collected in 1997, 2000 and 2004. Analysis was performed on proportions of mixed genotype infections, allelic diversity, population differentiation, effective population size and bottleneck effects.
We have found high levels of genetic diversity and minimal inter-population genetic differentiation typical of African continental regions with intense and stable malaria transmission.
We detected significant differences between the years, with special emphasis for 1997 that showed the highest proportion of samples infected with *P. falciparum* and the highest mean number of haplotypes per isolate.
This study establishes a comprehensive genetic data baseline of a pre-intervention scenario for future studies; taking into account the most recent and successful control intervention on the territory.

1. Introduction

Malaria remains the main infectious parasitic disease in the world with 214 million cases/year, affecting mainly children under 5 years-old and causing 438,000 deaths (WHO, 2015). Most cases occur in sub-Saharan Africa and almost all of the malaria-attributed deaths are caused by the parasite *Plasmodium falciparum*, the most prevalent malaria parasite in Africa (WHO, 2015).

Until 2000, most of the genetic epidemiology studies on *P. falciparum* were based on functional or adaptive genetic markers (Day et al., 1992; Meyer et al., 2002). Due to selective forces, these loci are more prone to mask transmission patterns. That is why selectively neutral loci are essential when investigating population changes. When possible, a general population structure approach with neutral loci should precede the analysis of genes of interest (i.e. functional or adaptive) and provide a convenient framework for the later approach (Gauthier and Tibayrenc, 2005).

Neutral microsatellite loci have shown a range of population structures in *P. falciparum* linked with the different transmission and endemicity scenarios (Anderson et al., 2000a). These range from high gene flow levels in high transmission areas such as Africa (Conway et al., 1999; Mu et al., 2005; Mobegi et al., 2012) to fragmented population structure in some low transmission areas in South America (Machado et al., 2004) and Asia (Anthony et al., 2005; Iwagami et al., 2009; Pumpaibool et al., 2009).

São Tome and Principe (STP), a nation comprising an archipelago in the Gulf of Guinea (Fig. 1), has suffered the burden of malaria since the 16th century. Until the last decade, malaria was the major cause of morbidity and child mortality in the islands reaching meso- to hyper-endemic levels (Pinto et al., 2000a). The four human malaria parasites have been recorded in the archipelago although *P. falciparum* was much the commonest, being found in 96.8% of positive cases and all mixed infections sampled in 1997 (Pinto et al., 2000a; Pinto et al., 2000b), and 99–100% between 2010 and 2014 (WHO, 2015).

In the early 1980's, a malaria eradication program was implemented by combining indoor residual spraying (IRS) with DDT and chloroquine (CQ) treatment and prophylaxis. While it lasted, malaria prevalence was reduced to 0.6%, and mortality down to zero. In 1983, the eradication program was interrupted due to financial and political constraints. This event triggered an epidemic in 1985–1986 and malaria prevalence rebounded to former levels (Ceita, 1986; Baptista, 1996; Loureiro et al., 1996). Meanwhile, CQ resistance in the parasite, had expanded throughout the archipelago (Lopes et al., 2002). Thus, in the beginning of the new millennium malaria incidence in STP was 40–50%, the first line of treatment was still CQ and the combination of sulfadoxine and pyrimethamine (SP) was the second line (OMS and UNICEF, 2003).

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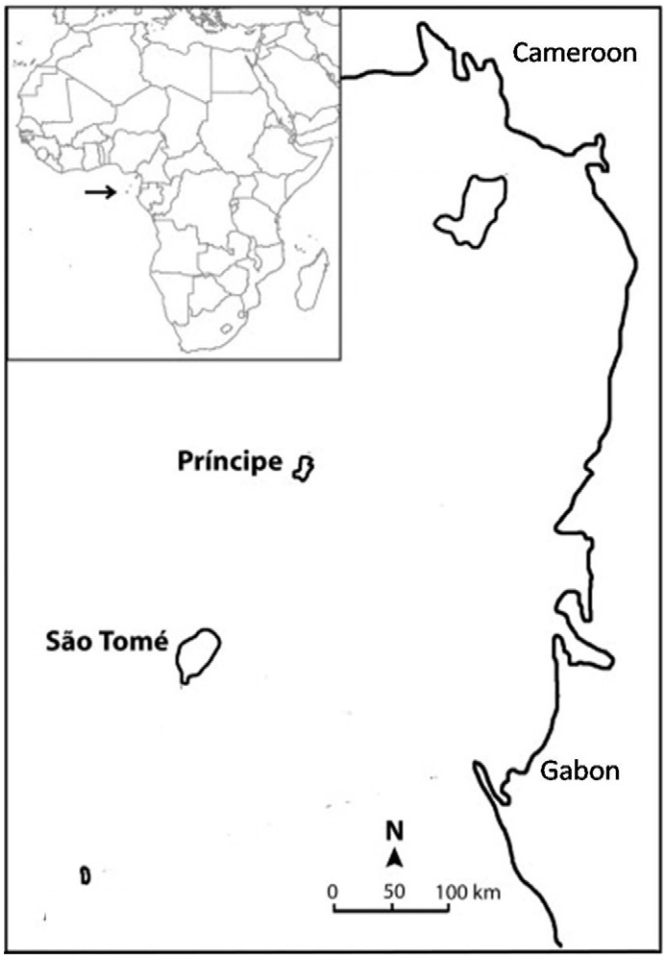

Fig. 1. Map of São Tomé and Príncipe islands.

In the mid-2000s, the STP Ministry of Health with the support from the Taiwanese Government initiated a new integrated malaria control plan (Lee et al., 2010a; Lee et al., 2010b; Lopes, 2013). This included prompt diagnosis and early treatment with artemisinin derivatives (artesunate and amodiaquine) as first line. In addition with vector control using IRS and long-lasting insecticidal nets (Centro Nacional de Endemias, 2004). The combination of SP was only used for intermittent preventive therapy during pregnancy (Salgueiro et al., 2010). The measures applied were so successful that a decline of 95% of malaria morbidity and mortality was observed between 2004 and 2008. However in 2009, malaria morbidity tripled and mortality doubled in children less than five years old. After an emergency intensification of the on-going program, malaria incidence diminished back to less than 1% (Lee et al., 2010a). Since then, STP has become a low transmission country and one of the few nations with decreases of more than 90% in malaria admission and death rates (WHO, 2015) and a pre-elimination scenario for the island of Principe (Lee et al., 2010b). The number of malaria cases reported in 2014 was 1754, and zero deaths (WHO, 2015).

The only malaria vector present in the archipelago of STP is *Anopheles coluzzii*, formerly known as the M molecular form of *Anopheles gambiae* s.s. (Coetzee et al., 2013). Previous studies showed marked zoophilic, exophilic/exophagic habits of these island populations in contrast to the behaviour described for this species in mainland Africa (Sousa et al., 2001). Genetic analyses revealed significant population differentiation in the vector within and between islands (Pinto et al., 2002, 2003). Furthermore, this study suggested that vector control conducted in the 1980s based on DDT did not affect the effective population size (Ne) of the mosquito vector. This led to the hypothesis that, rather than vector population reduction, the anti-parasitic drugs used in the eradication program coupled with the interruption of human-mosquito contact may have led to the decrease of malaria prevalence observed in STP in the 1980s.

In this study we analyzed the genetic diversity levels of microsatellite loci in *P. falciparum* samples from STP in three main years of the last decades (1997, 2000 and 2004). Our main goals were: (1) to examine the levels of genetic variation and population differentiation; (2) to estimate the effective population size and detect eventual population perturbations; (3) to compare those parameters over time and space.

2. Materials and methods

2.1. Study area and sample collection

Located 240 km northwest of Gabon, West Africa, São Tomé and Príncipe (STP) is an archipelago composed of two main islands: São Tomé (859 km^2) and Príncipe (142 km^2) (Fig. 1). Most of the population (150,000) lives in the main island of São Tomé. There are two dry seasons, a long one from June till August and a shorter one in January, when rainfall is reduced but rarely absent (Pinto et al., 2003).

Blood samples were obtained by finger prick in 1997 (Pinto et al., 2000a 2000 and 2004) (Salgueiro et al., 2010). The number of malaria cases reported in the collection years was: 47,757 in 1997, 43,488 in 2000 and 53,917 in 2004 (WHO, 2008).

In Table 1, we detailed the year, month, site of collection and sizes (N) of the blood samples used in the present study. Collections were made as part of active-case malariological surveys by the Centro Nacional de Endemias, Ministry of Health of STP, who provided ethical clearance for the study. No age restrictions were applied for the samples collected in 1997 and 2000. Conversely, the collection from 2004 was carried out only in children up to nine years old. Informed verbal consent was obtained from all adult subjects. Parents or tutors responded on behalf of children. Individual blood spots were maintained on Whatman No. 4 filter paper at room temperature until further processing.

2.2. DNA isolation

DNA was extracted with a Saponin/Chelex protocol (Plowe et al., 1995). *P. falciparum* infections were identified by a nested-PCR reaction (Snounou et al., 1993). Only isolates positive for *P. falciparum* were used in the subsequent analysis.

2.3. Microsatellite genotyping

Twelve microsatellite loci were used in this study. The names (and chromosome locations) (Su et al., 1999) of the markers are: TA81 (Chr5), TA109 (Chr6), TA87 (Chr6), TA42 (Chr5), PfPK2 (Chr12), PfG377 (Chr12), TA1 (Chr6), TA40 (Chr10), Polyα (Chr4), TA60 (Chr13) ARAII (Chr11), and TA102 (Chr12). Primer sequences and PCR conditions are described in (Anderson et al., 1999; Conway et al., 2001; Greenhouse et al., 2006).

Amplified products were run on an automatic sequencer (ABI 3730, Applied Biosystems) and sizes scored with the GENEMARKER software (SoftGenetics). Only samples that successfully amplified at no less than six loci were included in the data analysis (Table 1).

2.4. Data analysis

We scored all the alleles at a given locus if minor peaks were more than one-third the height of the predominant peak. Analysis was performed on proportions of mixed infections by measuring the number of haplotypes detected in an isolate, defined as the maximum number of alleles scored at the locus with the highest number of alleles (Mobegi et al., 2012) (Appendix I). The multiplicity of infection (MOI, i.e., the number of parasites genetically distinguishable by different