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# Accelerating towards malaria elimination in South East Asia : identifying and delineating residual pockets of transmission

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# Sorbonne Université

Ecole doctorale Pierre Louis - Santé publique à Paris : épidémiologie et  
sciences de l'information biomédicale

*Unité « Malaria : Parasites et hôtes », Institut Pasteur*

## **Accelerating towards malaria elimination in South East Asia: Identifying and delineating residual pockets of transmission**

Présentée par

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Thèse de doctorat d'épidémiologie

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*Dedication*

To my love, Isa and Nea

To my parents Ina and Lothar

To the countless people who suffered pain or loss due to malaria

## Acknowledgements

Without the enormous support of my partner Isabelle this thesis would not have been possible. Isa, thank you for the load you took off my back. I apologize to you and Nea, as you would have deserved every minute of attention that has gone into this work instead.

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## Abstract

After decades of global malaria reduction, some countries are now aiming to eliminate this disease. The countries of the Greater Mekong Subregion committed to elimination by 2030. With intensified elimination efforts, malaria transmission becomes fragmented, and high incidence is restricted to remote areas. Some groups are particularly exposed, e.g. forest goers in Cambodia. Risk patterns must be well understood to tailor interventions to the local context. Missed milestones indicate the need for more efficient elimination programmes.

This thesis set out to delineate risk heterogeneity in a remote, high-incidence part of Cambodia. Across villages along a gradient of proximity to the forest, we stratified the risk of PCR-detectable infections by gender, age, forest-going, and residence inside or outside the forest. We applied established tools for spatial cluster detection and introduced the spatial signature method to malaria epidemiology, i.e. determining the prevalence within increasing distance to index infections. This way, we also assessed spatial and spatiotemporal clustering of infections in observational studies from Brazil, Thailand, Solomon Islands, and Senegal.

In Cambodia, we demonstrated that pockets of high prevalence of *Plasmodium spp.* infections persist in the country, predominated by *P. vivax* and asymptomatic, sub-patent infections. This burden can decrease to near zero within a few kilometres of forested areas. While forest work by male adolescents and adults is the strongest behavioural driver of infection risk, in villages inside the forest the whole population is affected. Particularly in Cambodia, Brazil, and Thailand, infections increasingly clustered closer to other infections in space and time. Clustering tended to intensify at lower overall prevalence in the study area.

Given remaining pockets of high transmission, Cambodia has scaled up its village and mobile malaria worker programme. The country has also finally started implementing radical cure against *P. vivax* relapses. However, rapid diagnostic tests and passive case detection will continue to miss most asymptomatic, sub-patent infections. The more homogeneous risk in forest goers and villages could justify mass drug administration or testing and treatment. The clustering of infections in a few hundred metres argues for reactive approaches that include neighbouring households. In summary, this thesis improved the understanding of persisting malaria and offered new tools that hopefully help to accelerate towards malaria elimination.

## Résumé

Après plusieurs décennies marquées par un déclin global du paludisme, les pays de la sous-région du Grand Mékong se sont engagés à l'éliminer d'ici 2030. Avec l'intensification des efforts d'élimination, les zones de forte incidence du paludisme se restreignent aux régions reculées. Certaines populations sont particulièrement exposées, notamment lors de déplacements en zones forestières. Le risque d'infection doit être bien décrit afin d'adapter localement les stratégies d'intervention. Les retards par rapport aux objectifs prévus d'élimination confirment la nécessité d'améliorer les programmes de contrôle.

Cette thèse a eu pour but de décrire et comprendre l'hétérogénéité du risque d'infection dans une région reculée du Cambodge. Nous avons stratifié le risque, en exploitant des covariables démographiques et géographiques, et avons appliqué des outils existants pour la détection de clusters. Nous avons introduit la notion de signature spatiale de la transmission pour l'épidémiologie du paludisme. A partir de cas index confirmés par PCR, nous avons décrit l'évolution de la prévalence en fonction de la distance aux cas, et avons étendu cette analyse aux études menées au Cambodge, au Brésil, en Thaïlande, aux Îles Salomon et au Sénégal.

Au Cambodge, nous avons démontré que des poches de prévalence élevées d'infections à *Plasmodium spp.* subsistent, majoritairement causées par des infections de *P. vivax*, asymptomatiques et difficiles à détecter en routine. La zone d'influence de ces poches ne dépasse pas quelques kilomètres en bordure des zones forestières. Le travail en forêt est le facteur de risque le plus important à l'échelle de la population, et le risque s'étend à l'ensemble des habitants des villages forestiers. Au Cambodge, au Brésil et en Thaïlande, les cas index et secondaires sont très proches, géographiquement et dans le temps.

Le Cambodge a intensifié son programme de lutte à l'échelle locale et introduit le traitement « radical » des infections récurrentes à *P. vivax*. Cependant, la détection passive des cas continuera à manquer la plupart des infections. Le risque homogène dans les villages forestiers pourrait justifier des campagnes de distribution massive d'antipaludéens. Le faible rayonnement géographique des clusters plaide en faveur d'approches réactives restreintes aux foyers avoisinants. En résumé, cette thèse a amélioré la compréhension du paludisme résiduel et propose de nouveaux outils qui aideront à son élimination.

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## List of abbreviations

ACT	Artemisinin-based combination therapy
G6PD	Glucose-6-phosphate dehydrogenase
GMS	Greater Mekong Subregion
GTS	Global Technical Strategy for malaria 2016-2030
IRS	Indoor residual spraying
ITN	Insecticide-treated bed net
LLIN	Long-lasting insecticide-treated net
LM	Light microscopy
MDA	mass drug administration
MMW	Mobile malaria worker
NMP	National Malaria Programme
RDT	Rapid diagnostic test
VMW	Village malaria worker
WHO	World Health Organization

## Chapter 1: General introduction

Chapter 1 recaps the key facts of malaria, its biology, and epidemiology. It outlines global progress towards malaria elimination and challenges, zooming in on South East Asia.

### 1.1. Malaria and *Plasmodium spp.* infection

Malaria is caused by infection with *Plasmodium spp.* parasites, mainly of five species: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (Heymann, 2008; Lee et al., 2022). The parasites are transmitted via bites by female *Anopheles spp.* mosquitoes. *P. falciparum* and (outside of Sub-Saharan Africa) *P. vivax* are the most prevalent species. *P. ovale* and *P. malariae* are less prevalent (Mueller, Zimmerman, & Reeder, 2007). *P. knowlesi* usually infects non-human primates, but zoonotic infections are occasionally (and increasingly) reported from inhabited forested regions in South East Asia and the Western Pacific (Lee et al., 2022).

Malaria is a febrile illness that manifests with other non-specific symptoms, including chills, myalgias, and headaches. A few days later, many cases develop anaemia, splenomegaly, and thrombocytopenia. Severe malaria may occur, e.g. cerebral malaria characterized by acute encephalopathy, and is – if not treated promptly and appropriately - fatal in many cases (Heymann, 2008). Infections with *P. falciparum* entail the highest risk for severe and fatal disease. *P. vivax* malaria has often been referred to as ‘benign’ but can cause chronic anaemia and sometimes severe fatal disease, particularly in children (Douglas et al., 2013; Mueller et al., 2009).

The part of the parasite lifecycle in the human begins when *Anopheles spp.* mosquitoes inoculate *Plasmodium spp.* sporozoites into the human skin. These sporozoites then reach hepatocytes through the bloodstream. Multiple rounds of mitotic nuclei replication lead to the schizont form, which sequesters thousands of merozoites back into the bloodstream. These invade erythrocytes, form trophozoites and multiply into merozoites in a further round of schizogony. Some merozoites differentiate into gametocytes, the lifecycle stage infective to blood-feeding *Anopheles spp.* In the mosquito, the parasite undergoes sexual reproduction, replicates in oocysts, and forms sporozoites. They migrate to the mosquito salivary gland, from where they can be transmitted onward.

In contrast to the other species, *P. vivax* and *P. ovale* sporozoites form dormant stages in hepatocytes called hypnozoites (Mueller et al., 2009). These re-activate and cause relapses, i.e. bloodstream infections weeks, months, or years after the initial infection by a mosquito (White, Shirreff, Karl, Ghani, & Mueller, 2016). Contrary to *P. falciparum*, *P. vivax* merozoites prefer reticulocytes, potentially causing chronic anaemia. Also, *P. vivax* gametocytes can develop prior to symptom onset (Adams & Mueller, 2017; Mueller et al., 2009).

Artemisinin-based combination therapies (ACTs) are the most effective treatment against uncomplicated malaria. These drugs treat the bloodstream parasitaemia of all human malaria pathogens and can cure *P. falciparum*, *P. malariae*, and *P. knowlesi*. However, they are ineffective against hypnozoites. To prevent relapses from *P. vivax* or *P. ovale* and thus achieve ‘radical cure’, 8-aminoquinolines such as primaquine are needed. However, primaquine can induce haemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, one of the most common enzyme deficiencies worldwide (World Health Organization, 2022).

## 1.2. Malaria epidemiology worldwide

Malaria occurs across many countries in Latin America, Africa, Asia, and Oceania, particularly in tropical and subtropical areas. It thus poses a risk of illness and death to billions of people and puts an additional economic strain on many resource-poor countries (World Health Organization, 2015a). The latest *World Malaria Report 2021* from the World Health Organization (WHO) estimated 241 million malaria cases and 627,000 deaths from malaria worldwide in 2020. Ninety-five per cent of these cases and 96% of malaria deaths occurred in the WHO African region, and 77% of all malaria deaths happened in children under five years (World Health Organization, 2021a).

Malaria case incidence varied across endemic areas from 233 per 1,000 population at risk in the WHO African region to 2 cases per 1,000 in the Western Pacific region in 2020. Likewise, mortality rates ranged from 62 deaths per 100,000 population at risk to 0.4 per 100,000, respectively. However, low-incidence regions can enclose high-incidence countries, e.g. Papua New Guinea in the Western Pacific region, with 164 cases per 1,000 (World Health Organization, 2021a). Generally, the heterogeneity of malaria risk is ‘fractal’, i.e. low-incidence

world regions include high-incidence countries, low-incidence countries contain high-incidence provinces, and low-incidence provinces comprise high-incidence villages (Lana et al., 2021). Risk diversity extends further, e.g. to the levels of households and individuals within villages (Smith, McKenzie, Snow, & Hay, 2007; Woolhouse et al., 1997).

Cases in sub-Saharan Africa are almost exclusively caused by *P. falciparum*. In contrast, *P. vivax* causes a considerable share of cases in the other malaria-endemic WHO regions, e.g. an estimated 30% of all cases were *P. vivax* mono-infections in the Western Pacific in 2020. In the Americas, even 75% of cases were attributed to *P. vivax* mono-infections (World Health Organization, 2021a). In particular, in countries that are co-endemic with *P. falciparum* and *P. vivax*, it has frequently been observed that as malaria case incidence decreased with intensified control interventions, the proportion of cases attributable to *P. vivax* increases (Price, Commons, Battle, Thriemer, & Mendis, 2020), see also Figure 2 on page 13.

### 1.3. The goal of malaria elimination

Since the United Nations included the halt of the spread of malaria in the *Millennium Development Goals* in 2000, the global incidence of malaria cases has been reduced by a quarter, i.e. from 81 cases per 1,000 population at risk in 2000 to 59 cases per 1,000 in 2020, see Figure 1. Malaria mortality has halved from an estimated 30 deaths per 100,000 population at risk in 2000 to 15 deaths per 100,000 in 2020 worldwide (World Health Organization, 2000).

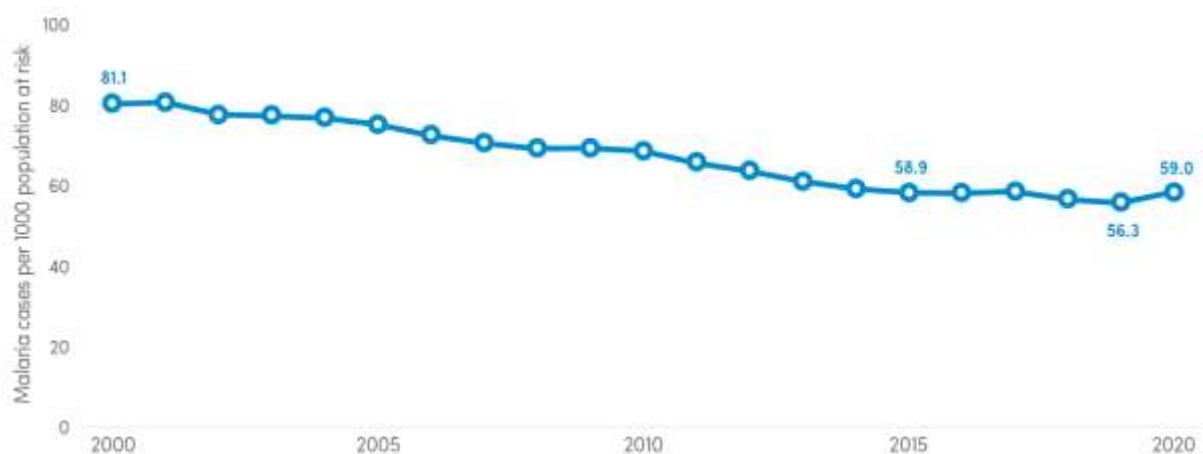


Figure 1 Global malaria case incidence (cases per 1,000 population at risk) from 2000 until 2020 as estimated by the WHO (World Health Organization, 2021a).

A massive scale-up of the major malaria control tools made this success in burden reduction possible. It mainly comprised insecticide-treated bed nets (ITNs), including long-lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS), rapid diagnostic tests (RDTs), and ACTs. Given this momentum, the Bill & Melinda Gates Foundation and the WHO called for malaria elimination in 2007 (World Health Organization, 2020). Until 2020, 23 countries had reached the WHO category ‘prevention of re-introduction’, i.e. had reported zero indigenous cases in a year (World Health Organization, 2007). After reporting no locally acquired cases for three years, a country is eligible for malaria-free certification by the WHO. 12 countries had achieved this status by 2020 (World Health Organization, 2021a), mainly from the Middle East or Central Asia.

The milestone of malaria elimination in 8-10 countries until 2015 was surpassed (The Roll Back Malaria Partnership, 2008), and the malaria-free certification of China and El Salvador in 2021 was a great success (World Health Organization, 2021a). However, the *Global Technical Strategy for malaria 2016-2030* (GTS) aimed to eliminate malaria from 10, 20, and 35 countries in which malaria was endemic in 2015 until 2020, 2025, and 2030, respectively (World Health Organization, 2015a). Six countries have reached the malaria-free criterion since 2016, so the intermediate 2020 target was missed. Overall, progress in reducing the malaria burden has levelled off globally (World Health Organization, 2021a).

South East Asia and especially the Greater Mekong Subregion<sup>1</sup> (GMS) has attracted particular attention since 2011 regarding malaria elimination due to the detection of resistance to artemisinin derivatives in *P. falciparum* at the Cambodia-Thailand border (Carrara et al., 2009; Dondorp et al., 2009; Noedl et al., 2008; Noedl, Socheat, & Satimai, 2009; Vijaykadga et al., 2006; World Health Organization, 2013). Except for China, which reports zero cases since 2017, malaria remains endemic across the GMS, with high malaria incidence and mortality observed until recently in Cambodia (World Health Organization, 2021a, 2021b). In the WHO *Strategy for malaria elimination in the Greater Mekong Subregion 2015-2030*, all GMS countries committed to eliminating *P. falciparum* in areas where multidrug resistance is known by 2020, all *P. falciparum* malaria by 2025 and all malaria by 2030. Some countries even declared earlier targets, e.g. elimination of *P. falciparum* malaria overall by 2020 and

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<sup>1</sup> including Cambodia, China (Yunnan province), Laos, Myanmar, Thailand, and Vietnam

complete malaria elimination by 2025 in Cambodia (Cambodian Ministry of Health, 2011; Chinese Ministry of Health, 2010; World Health Organization, 2015b).

#### **1.4. Challenges to elimination and the need to accelerate**

Several recent developments threaten global elimination efforts. They include the spread of drug-resistant parasites, particularly in *P. falciparum*, and strains that evade common RDT target antigens or more efficient and resilient vector species (Faulde, Rueda, & Khaireh, 2014; Gamboa et al., 2010; World Health Organization, 2015b, 2021a).

In addition, many countries, particularly those from the GMS, face challenges due to inherent features of malaria epidemiology. First, a large share of asymptomatic infections which are not detected during routine, curative case management. Second, the predominance of *P. vivax* infections entailing transmission prior to symptoms, difficult cure, and relapses. Third, the overall geographic fragmentation of malaria transmission with persistently high infection levels in remote areas. While the emerging threats render malaria elimination a race against time, the inherent challenges of malaria epidemiology hinder the progress of (inter-)national control programmes. Those roadblocks must be overcome if the ambitious elimination timeline is to be met.

This thesis is embedded in the Asia-Pacific International Center of Excellence in Malaria Research (ICEMR) project. It applies state-of-the-art and novel molecular, immunological, entomological, and modelling techniques to better understand malaria transmission in Papua New Guinea and Cambodia (Mueller et al., 2022). Regarding Cambodia, it especially examines the dynamics of forest-based transmission and *P. vivax* malaria and their implications for malaria elimination (Robinson et al., 2022).

This thesis assesses the high levels of asymptomatic and *P. vivax* infections and spatial and social heterogeneity in infection risk based on a cross-sectional survey in a remote area in Cambodia. The work introduces a novel approach to describe spatial and temporal clustering of infections and applies it to data from Cambodia, Thailand, Solomon Islands, Brazil, and Senegal. The thesis compares it to established spatial statistical tools and reviews the findings in light of their utility to help accelerate towards malaria elimination.

## Chapter 2: State of the art

Chapter 2 recaps the current knowledge of the progress towards malaria elimination in South East Asia. It lists the presently recommended public health interventions. A focus lies on the challenge of fragmented malaria burden and statistical tools for spatial risk patterns.

### 2.1. Progress towards malaria elimination in South East Asia

Over the past two decades, countries in the GMS have made enormous progress in reducing the malaria burden. Annual numbers of indigenous cases of *P. falciparum* malaria have fallen by 93% from 2000 until 2020 and overall malaria case numbers by 78%, see Figure 2, despite a drastic increase in case numbers between 2006-2012. Coordinated efforts against artemisinin-resistant *P. falciparum* helped to change the course and initiate a decade of even more marked reductions in case numbers (World Health Organization, 2021a).

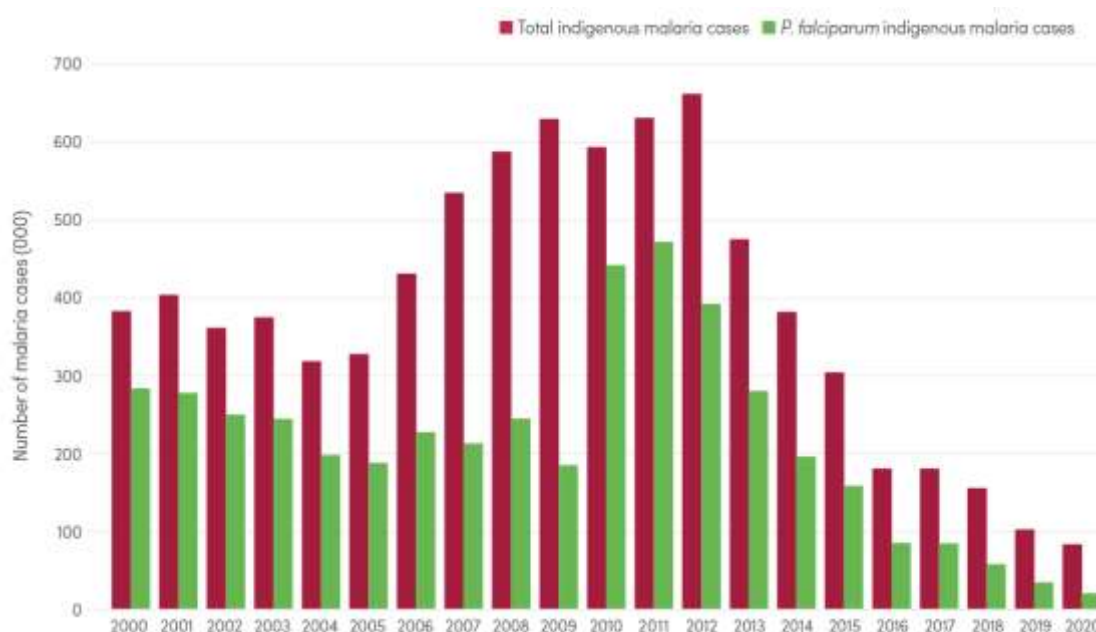


Figure 2 Annual case numbers of indigenous overall and *P. falciparum* malaria cases in the GMS from 2000 until 2020 (World Health Organization, 2021a).

Reductions of 60% or more in malaria incidence and mortality were achieved in all GMS countries. The 2020 GTS milestone of a 40% reduction was thus met (World Health Organization, 2015a, 2021a). In Cambodia, reported case numbers per year have declined drastically over the last 20 years, from 129,167 cases and 608 deaths in 2000 to 4,279 cases

and zero deaths (for four sequential years) in 2021, see Figure 3. Laos and Vietnam also reported zero malaria deaths in 2020.

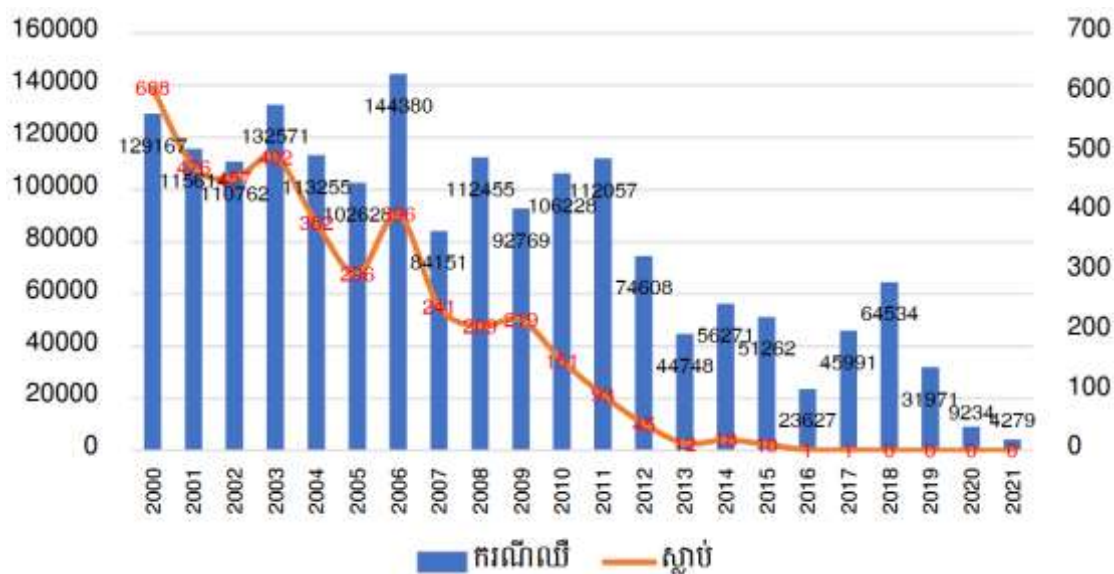


Figure 3 Malaria case numbers (blue bars, left axis) and deaths (orange line, right axis) as per the Cambodian control programme (Cambodian National Center for Parasitology, 2021).

An increase in incidence in 2017-2018 was linked to the emergence of piperazine-resistant *P. falciparum* and the suspension of the village malaria worker (VMW) programme (see the following section) 2014-2017 (Roesch et al., 2020; Sovannaroth et al., 2022). After changing the first-line treatment from dihydroartemisinin-piperazine to artesunate-mefloquine in 2017 and restoring the VMW programme, *P. falciparum* cases have decreased since 2018 and malaria cases overall since 2019 (Cambodian National Center for Parasitology, 2021; Mekong Malaria Elimination Programme, 2019).

Cambodia reported 10% of cases caused by *P. falciparum* in 2020 and thus missed the target of *P. falciparum* elimination by that year (World Health Organization, 2015b). Considering unreported cases, the WHO estimates 69,136 malaria cases in Cambodia in 2020, which sets malaria elimination far from reach.

## 2.2. Toolbox for malaria elimination

The tools that the WHO particularly recommends for the prevention of infection include: 1) distribution of ITNs/LLINs free of charge, 2) IRS if vectors feed and people sleep mainly indoors, 3) preventive chemotherapies irrespective of diagnosis, either targeting risk



groups or population-wide via mass drug administration (MDA), and 4) RTS,S/AS01 vaccination against *P. falciparum* malaria for children (World Health Organization, 2022).

Given the unspecific symptoms, treatment against malaria should follow confirmation via light microscopy (LM) or RDTs. Uncomplicated *P. falciparum* malaria should be treated with ACT daily over three days. In low-transmission settings, a combination with a single primaquine dose is recommended without needing G6PD testing to lower the risk of onward transmission by killing gametocytes. Uncomplicated malaria by any of the other *Plasmodium spp.* should be treated with ACT (or chloroquine unless the area is known for resistance). *P. vivax* and *P. ovale* should be treated with primaquine daily for 7-14 days to prevent relapses if they are not G6PD deficient (World Health Organization, 2022).

The geographic heterogeneity of malaria poses strategic and logistical challenges to national malaria programmes (NMPs). Thus, the WHO recommends regionally stratified interventions to tailor elimination interventions to the local distribution of the remaining reservoir of infection and to target high-risk groups, see Figure 4 (Global Malaria Programme, 2017; World Health Organization, 2015a, 2022).

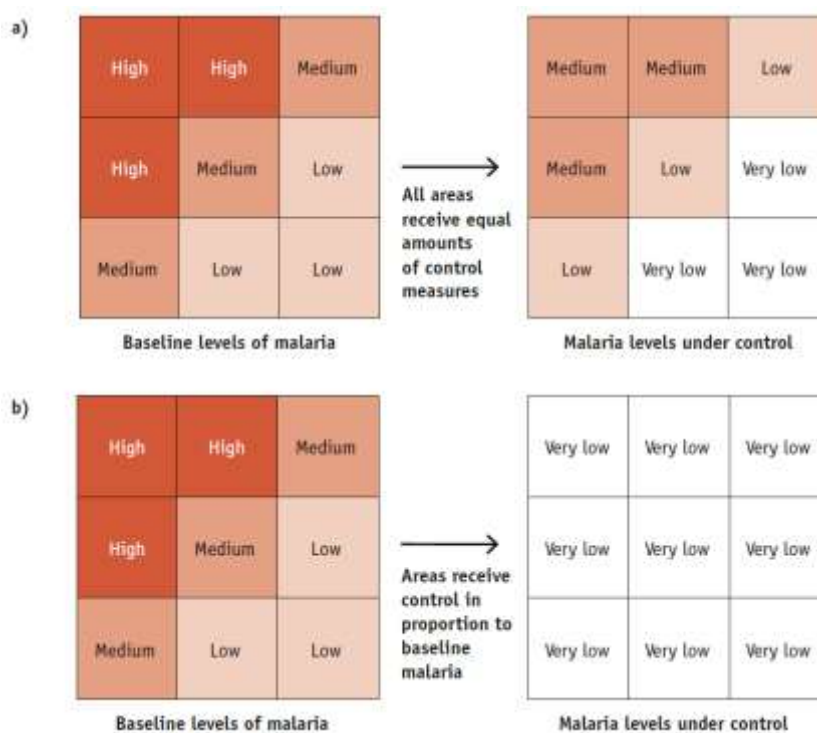


Figure 4 The effect of (a) an untailed intervention programme compared to (b) a programme that stratifies interventions by transmission levels (World Health Organization, 2014).

In delimited areas with transmission across the general population, ‘mass’ interventions may be considered. When transmission is (largely) confined to certain high-risk groups, ‘targeted’ interventions could be applied to group members before, during, or following the exposure. ‘Reactive’ interventions can be initiated upon the (usually passive) detection of cases: cases are followed-up, and interventions are also targeted to co-exposed contacts or people residing with or close to the index case. In all three categories, interventions could be presumptive drug administration or testing and treatment.

The Cambodian NMP has mainly tackled malaria through the cost-free distribution of ITNs/LLINs and effective diagnosis by LM or RDTs and treatment with ACT in health centres and more remote health posts (Maude et al., 2014). Access to diagnosis and treatment for remote populations was further improved by the VMW programme, launched in 2004 (Canavati et al., 2016). These community health workers provide cost-free malaria diagnosis by RDTs, ACT, and behaviour change communication to the general population in remote villages. In addition, mobile malaria workers (MMWs) were established to target migrant populations, e.g. mobile groups that stay in the forest for extended periods for occupational activities. IRS and vaccination are not adopted in Cambodia. Until 2019, all RDTs or LM-confirmed infections had been treated with ACT. Primaquine treatment for *P. vivax* infections and prior G6PD testing was piloted in 2019-2020 and has been scaled up countrywide since then (Sovannaroeth et al., 2022; World Health Organization, 2021a, 2021b).

## **2.3. Regional challenges to malaria elimination**

### **2.3.1. Predominance of *P. vivax***

In the past, *P. vivax* was reported to cause a minority of cases in countries across the GMS (World Health Organization, 2021a). To date, the share of cases with *P. vivax* infections has increased in all countries, and in most, *P. vivax* now accounts for most of the cases: 42.4% of cases in Vietnam, 54.9% in Laos, 74.1% in Myanmar, 89.5% in Cambodia, and 93.5% in Thailand in 2020 (World Health Organization, 2021a).

Case management with ACT after RDT or LM diagnosis (in combination with prevention measures through ITNs and IRS) has proven very successful in reducing case incidence.

However, this success has been more pronounced against *P. falciparum* than against *P. vivax* (Price et al., 2020; World Health Organization, 2021a). *P. vivax* infections lead mostly to low-density, asymptomatic infections that can be hard to detect by RDTs or even LM. Several relapses per primary *P. vivax* infection lead to partial immunity more rapidly and increase the likelihood of a milder or asymptomatic course upon relapse or new primary infection (Mueller et al., 2009). Thus, clinical case management that relies on patients' self-reporting to health facilities targets patients with acute *P. falciparum* infections more effectively than those with a *P. vivax* infection. Furthermore, primaquine treatment of *P. vivax* cases is often not established in endemic countries because of prevalent G6PD deficiency in the population, the risk of drug-induced haemolysis, and because G6PD testing prior to treatment is costly. In addition, the daily dosing over 14 days reduces adherence compared to the 3-day ACT-only regimens. A further challenge to *P. vivax* control is the early production of gametocytes prior to symptom onset (Adams & Mueller, 2017; Mueller et al., 2009), whereas *P. falciparum* gametocytes are more easily hit by gametocidal treatment as they appear only days to weeks after clinical manifestation (Lover, Baird, Gosling, & Price, 2018).

### **2.3.2. High prevalence of asymptomatic infections**

The malaria incidence reported by NMPs is mainly based on clinical cases who consult health facilities. The measure thus misses patients who do not attend such facilities, be it due to mild symptoms or because they frequent traditional healers (Chatterjee, 2005). Patients from remote parts of a country, far from the nearest health facility, may also be missed. VMWs, MMWs, or health posts in the periphery of Cambodia may lower but not abolish such barriers. Furthermore, the detection threshold of LM and RDTs does not allow the detection of sub-patent parasitaemia in mildly symptomatic patients. Lastly, asymptomatic individuals will not consult health facilities in the first place. Sub-patent or asymptomatic infections will be particularly prevalent in areas or populations with high transmission levels (due to immunity). Rates of infections will thus exceed incidence, even more so in remote areas with high transmission levels, including the north-eastern provinces in Cambodia (Stresman et al., 2020).

The disproportionately stronger divergence of reported incidence and actual infection rates in high-transmission areas make field studies in these areas instrumental, as they actively

sample asymptomatic people. The first survey determining infection levels in Cambodia's general population was conducted by Incardona *et al.* in three high-incidence provinces in peak transmission periods in 2001-2003 (Incardona *et al.*, 2007). Comparing LM-derived prevalence to reported case incidence, the discrepancy was between 5-fold in Sampovloun and higher than 100-fold in Koh Kong. That infection prevalence in surveys exceeded NMP records of clinical incidence was equivalently observed in other GMS countries, e.g. at the Thailand-Myanmar border in 2012 (Baum *et al.*, 2015) or in Myanmar (O'Flaherty *et al.*, 2021).

Asymptomatic and sub-patent infections often harbour parasitaemia levels that allow the transmission to mosquitoes and thus contribute to the persistence of malaria despite malaria control interventions (Kiattibutr *et al.*, 2017; Tadesse *et al.*, 2018). It is thus crucial to identify and treat this silent reservoir of infections if malaria elimination is to be achieved.

## **2.4. Geographic heterogeneity in malaria burden**

The countries in the GMS show a highly heterogeneous profile of malaria burden. At the lower end, China has had no locally acquired cases since 2017. In terms of incidence of indigenous malaria cases in 2020 in the other countries, the WHO estimates range from <0.1 per 1,000 population at risk in Vietnam and 0.2 per 1,000 in Thailand, and up to 5.8 per 1,000 in Cambodia. The malaria mortality estimates range from <0.1 per 100,000 population at risk in 2020 in Vietnam and Thailand, and up to 0.4 per 100,000 in Cambodia.

Within the countries, the malaria burden is also distributed highly unevenly, see Figure 5. In Thailand, large parts of the country are malaria-free. Areas with ongoing transmission are mainly restricted to the borders with regions in Myanmar, Malaysia, Laos, and Cambodia that have a relatively high incidence (World Health Organization, 2021e). Also, in Vietnam, low-level transmission mainly remains along the border with Cambodia and Laos. By contrast, in Laos, Myanmar, and Cambodia, parts of the country with high or very high transmission remain. While the mountainous northern half of Laos is mostly free of malaria, high transmission levels occur throughout the country's south and southeast along the border with Thailand with its plains along the Mekong. In Myanmar, provinces with no or low-level transmission can be found in the central lowlands, but the largest part of the country is covered with high transmission areas (World Health Organization, 2021c, 2021d, 2021f).



of people exposing themselves to forested areas temporarily, e.g. slash and burn farmers and loggers, but also miners, security forces, and dam construction workers. (Dysoley et al., 2008; Hii & Rueda, 2013; Singhanetra-Renard, 1993; World Health Organization, 2015b).

## 2.5. Statistical tools for low-scale spatial risk clustering

One way to delineate where remaining transmission occurs is to associate disease or infection risk on different spatial scales with increasing granularity, e.g. villages, hamlets, or individual households. Bannister-Tyrrell *et al.*, for example, conducted a cross-sectional survey in three villages in Ratanakiri province, north-eastern Cambodia, in 2016-2017. They found that odds for *Plasmodium spp.* infection were sufficiently explained by household-level information by contrast to individual or village-level covariates, suggesting the household as the unit of risk clustering (Bannister-Tyrrell et al., 2018).

Various methods are at hand for detecting spatial clustering of observations, e.g. malaria cases or infections. Abundant options are statistical tests for spatial autocorrelation, e.g. *Moran's I* or *Geary's C*, or *space-time statistics*. Based on geo-located households of reported malaria cases in Haiti in a low-transmission setting in 2014-2015, a study detected several areas of 1 km<sup>2</sup> in size with significant case clustering via a spatial autocorrelation statistic for cluster detection, called *Getis-Ord Gi\**, and overlapping space-time clusters via space-time permutation modelling (Dimer et al., 2021). Three cross-sectional surveys in northern Guatemala at low malaria endemicity in 2000-2001 found clusters of malaria cases by *Moran's I* and *Getis-Ord Gi\**, however highly variable in location over time (Malvisi, Troisi, & Selwyn, 2018). In the 1990s, the Trinidad and Tobago Ministry of Health was able to confirm *P. vivax* and *P. malariae* outbreaks and calculate their spatial extents via space-time statistics to adjust national outbreak management guidelines (Chadee & Kitron, 1999).

These days, the most popular method to locate malaria clusters is *spatial scanning*, particularly the *SaTScan™* implementation (Martin Kulldorff, 1997; M. Kulldorff & Nagarwalla, 1995). This method divides the study area (and for spatiotemporal clustering, potentially the study period in case of multiple time points of observations) into overlapping windows, e.g. circular areas of the area under study and time intervals during the study period, and varies the size of these windows. Within the windows, the number of observed vs. expected positive

observations are compared. The window with the maximum likelihood is considered the most likely cluster, often referred to as a 'hotspot'. The output of the calculation then is the location and size of that window, e.g. the circle on the map of the study area.

A cross-sectional survey in three villages in Cambodia's northeast, in Ratanakiri province in 2016, found clusters of *P. vivax*, *P. falciparum*, and *P. malariae* in sub-village areas (Durnez et al., 2018). In a cross-sectional survey along the border between French Guiana and Brazil at low prevalence of *P. falciparum* and *P. vivax* in 2017, hotspots could be located in remote neighbourhoods on village outskirts and small intra-village clusters (Mosnier et al., 2020). During its malaria elimination phase, China used SaTScan analyses on retrospectively collected and geo-coded malaria cases to identify remaining transmission clusters in Shandong province. The study identified several distinct clusters for indigenous and imported cases, which was helpful in guiding elimination interventions (Kong et al., 2017). In a low transmission area in South Africa, Mpumalanga province, SaTScan assisted in identifying clusters of confirmed malaria cases in seven towns over three years, 2002-2005 (Coleman et al., 2009). Based on cross-sectional surveys at the Thailand-Myanmar border in 2011-2012, Parker *et al.* found clusters of *P. vivax* infections in the dry season around a few year-round water sources (Parker et al., 2015).

Often when applied to observation time series, the SaTScan method finds hotspots, but these are not stable in time. For example, a cross-sectional survey with repeated data collection and sampling time points over two years in three villages in eastern Cambodia, Ratanakiri province, in 2016-2017 found hotspots in 2016. However, having lived in a hotspot in 2016 did not associate with a risk of *Plasmodium spp.* infection in 2017, whereas having lived in a household with a malaria case did associate with risk in the following year (Bannister-Tyrrell et al., 2019). Based on repeated cross-sectional surveys three months apart from 2013-2014 in three villages in western Cambodia, hotspots of *P. vivax* and *P. falciparum* infections were found, with clusters of subclinical infections close to clinical case clusters. However, hotspots were transient and not predictive of stable spatial clusters over time (Parker et al., 2017).

Based on the household locations of dengue fever patients in Bangkok over five years, Salje *et al.* developed a method to generalize the spatial structure of the underlying dengue

transmission dynamics (Salje et al., 2012). The authors also applied it to data on HIV and measles cases (Lessler, Salje, Grabowski, & Cummings, 2016). It made use of the knowledge that the viruses of some case pairs were more closely related than others, e.g. viruses of one of the four serotypes of the dengue virus. The method then infers spatial clustering by dividing the probability of case pairs being of the same serotype in a given distance interval by the probability of any other case pair being of the same serotype, regardless of distance, across moving distance intervals. An increasing relative probability of more closely related case pairs at shorter distances indicated spatial clustering, e.g. see Figure 6. Whereas SaTScan-based analyses aim to identify the locations of the clusters of infections (which may change over time), the spatial signature provides a description of the degree of clustering aggregated across a geographic area.

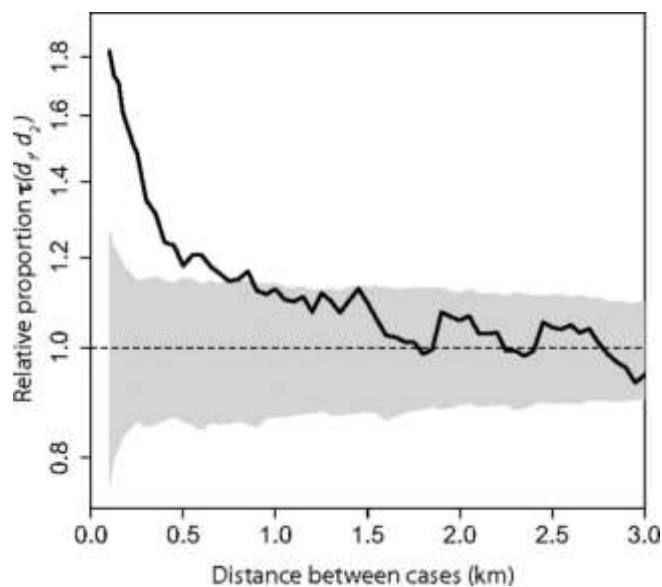


Figure 6 Spatial signature of dengue cases in the same month, i.e. the relative probability (y) of two case pairs being of the same serotype within a distance interval (x) compared to any other case pair carrying the same genotype. The ribbon indicates the 95% confidence interval as derived from a null distribution that erased any spatial clustering in time (Salje et al., 2012).




**Chapter 3: Forest malaria in Cambodia: the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in a cross-sectional survey in Mondulkiri province, Cambodia**

RESEARCH

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# Forest malaria in Cambodia: the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in a cross-sectional survey in Mondulhiri province, Cambodia

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## Abstract

**Background:** After a marked reduction in malaria burden in Cambodia over the last decades, case numbers increased again in 2017–2018. In light of the national goal of malaria elimination by 2025, remaining pockets of high risk need to be well defined and strategies well-tailored to identify and target the persisting burden cost-effectively. This study presents species-specific prevalence estimates and risk stratification for a remote area in Cambodia.

**Methods:** A cross-sectional survey was conducted in 17 villages in the high-incidence province Mondulhiri in the dry season (December 2017 to April 2018). 4200 randomly selected participants (2–80 years old) were tested for *Plasmodium* infection by PCR. Risk of infection was associated with questionnaire-derived covariates and spatially stratified based on household GPS coordinates.

**Results:** The prevalence of PCR-detectable *Plasmodium* infection was 8.3% (349/4200) and was more than twice as high for *Plasmodium vivax* (6.4%, 268) than for *Plasmodium falciparum* (3.0%, 125,  $p < 0.001$ ). 97.8% (262/268) of *P. vivax* and 92.8% (116/125,  $p < 0.05$ ) of *P. falciparum* infections were neither accompanied by symptoms at the time of the interview nor detected by microscopy or RDT. Recent travels to forest sites (aOR 2.17,  $p < 0.01$ ) and forest work (aOR 2.88,  $p < 0.001$ ) were particularly strong risk factors and risk profiles for both species were similar. Large village-level differences in prevalence of *Plasmodium* infection were observed, ranging from 0.6% outside the forest to 40.4% inside. Residing in villages at the forest fringe or inside the forest compared to outside was associated with risk of infection (aOR 2.14 and 12.47,  $p < 0.001$ ). Villages inside the forest formed spatial hotspots of infection despite adjustment for the other risk factors.

**Conclusions:** Persisting pockets of high malaria risk were detected in forested areas and in sub-populations engaging in forest-related activities. High levels of asymptomatic infections suggest the need of better case detection plans and the predominance of *P. vivax* the implementation of radical cure. In villages inside the forest, within-village

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exposure was indicated in addition to risk due to forest activities. Village-level stratification of targeted interventions based on forest proximity could render the elimination efforts more cost-effective and successful.

**Keywords:** Forest, Occupational risk, Spatial, Vivax, Hotspots, Cambodia, Greater Mekong Subregion

## Background

In the last two decades, Cambodia has seen a marked decrease in numbers of reported malaria cases from approximately 140,000 cases in 1999 to 60,000 in 2018 [1]. Likewise, mortality by malaria steadily reduced, with zero deaths reported for the first time in 2018. Similarly remarkable reductions have been observed in the other countries of the Greater Mekong Subregion [2]. Since 2014, these countries have even pursued the goal of elimination of all malaria by 2030 [3].

This ambitious aim however faces several obstacles. After an all-time low of approximately 20,000 reported cases in 2016, numbers have increased again in 2017–2018 [1, 2]. While the annual case numbers increased for both *Plasmodium falciparum* and *Plasmodium vivax* in 2017, they declined for *P. falciparum* in 2018 but increased further for *P. vivax* [1, 3]. Consequently, *P. vivax* now accounts for almost three-quarters of all malaria cases in Cambodia [2, 4].

The national malaria control programme (NMCP) is based on mass distribution campaigns of long-lasting insecticidal nets (LLIN), diagnosis by light microscopy (LM) and rapid diagnostic tests (RDT) in the public health sector, RDT-based testing by village/mobile malaria workers (VMW/MMW), and treatment of blood stage infections with artemisinin-based combination therapy (ACT) [5]. The spread of multi-drug resistant *P. falciparum* malaria in Western Cambodia [6] might explain the resurgence of *P. falciparum* cases in 2017 and the change of first-line treatment from dihydroartemisinin-piperaquine to artesunate-mefloquine their subsequent decline in 2018. For *P. vivax*, such resistance is not reported. However, this species has dormant liver-stages called hypnozoites that escape acute blood stage treatment and cause relapsing infections. Therefore, although LLINs do reduce *P. vivax* transmission and ACT is effective against blood stage *P. vivax* infection, neither intervention targets the hypnozoite reservoir. Hence, there is a less marked effect of these interventions on *P. vivax* than *P. falciparum*.

The only licensed drug with hypnozoitocidal effect is primaquine, typically administered at 0.25 mg/kg daily over 14 days in Cambodia. The roll-out of *P. vivax* radical cure is part of the national treatment guidelines but has not yet been implemented because of concerns over the drug's potential haemolytic effect in patients with G6PD deficiency [7, 8]. In summary, the control and elimination

efforts of the NMCP are successfully targeting clinical *P. falciparum* malaria (understandably in the context of drug-resistant *P. falciparum* [6] in the country) but to a lesser extent to *P. vivax*.

Another obstacle to elimination is the high prevalence of asymptomatic infections [9–12]. Neither health facilities nor VMWs reach these asymptomatic carriers as they do not seek diagnosis or treatment. Although of lower infectivity [13, 14], they remain however a potential source of onward transmission.

Alongside the sustained reduction in countrywide incidence, malaria has also become increasingly fragmented, affecting fewer, usually remote provinces. The highest incidence of malaria in Cambodia and across the Greater Mekong Subregion is now particularly found in the North-Eastern provinces such as Ratanakiri and Mondulakiri [1, 3]. These settings are characterized by a high proportion of ethnic minorities and agricultural or wood-logging activities as the main income sources. Exposure to *Anopheles* mosquitoes and *Plasmodium* in Cambodia is understood to primarily occur in the forest rather than peri-domestically [15–17].

Together with the Western province Pursat, the highest incidence per province in 2018 was reported for Mondulakiri [1]. However, previous studies on population-level burden and risk of malaria in Cambodia focused on the country's West and North [12, 16–21] or the Eastern province of Ratanakiri [10, 11, 15, 20, 22, 23]. Here, using a cross-sectional survey conducted in a rural area of Mondulakiri in the dry season in 2018, estimates of the prevalence of *Plasmodium* infection are shown and key risk factors identified.

## Methods

### Study area and census

Seventeen villages were selected in the Kaev Seima district, Mondulakiri province, in the Kingdom of Cambodia. The rainy season in Cambodia normally runs from June to October with the high transmission period from June through December [1, 4, 24]. Approx. two-thirds of the population in Mondulakiri is comprised of national ethnic minorities, with the Phnong ethnic group comprising the largest proportion [25, 26]. In November–December 2017, a census was conducted by visiting each household in the 17 villages and collecting basic demographics of household participants such as age and gender. A person's household was defined as location of main residence in

the village according to adult members of the household or the village head. GPS coordinates were collected using Garmin® GPSMAP® 64s devices. When no adult household member could be found, demographic information was obtained from the village head's registry book.

### Cross-sectional survey

Based on the census, a random selection of households was drawn oversampling small villages to ensure sufficient coverage (Additional file 1: Table ST1). Selected households were visited from mid-December 2017 until mid-April 2018 and all household members aged 2–80 years who had resided in the study area for at least 3 months were invited to participate in the survey. Upon informed consent, a questionnaire on household variables was administered to the head of household or another adult household member by trained interviewers. A questionnaire on individual-level variables was administered to each consenting household member. Children were interviewed assisted by a parent (or rarely another caregiver in the absence of the parents). Data were collected on tablets and run through automated data quality checks within days after the interview. In case of missing data or discrepancies, the field team was informed for immediate resolution if possible. Finger-prick blood samples were collected as thick and thin film slides and in K<sup>+</sup>EDTA-microtainers. Participants were screened for symptoms, *i.e.* feeling sick or feverish on the day of interview, having felt feverish over the preceding two days, or having an axillary body temperature of at least 37.5 °C. Upon any indication, the participant was administered a standard malaria RDT (Malaria Ag P.f/P.v, Standard Diagnostics Inc., South Korea) and referred to a local health care provider for treatment if positive.

### Detection of infection

The microtainer blood samples collected at the interview site were stored in 4 °C ice boxes. At a field laboratory, they were separated into plasma and cell pellet and frozen at –20 °C. Following transport to the main laboratory at Institut Pasteur of Cambodia in Phnom Penh, cell pellets were stored at –20 °C and plasma at –80 °C. Infections with any of the four human *Plasmodium* parasites were determined by real-time PCR [27]. In case of a positive genus-specific result, qPCR specific for *P. falciparum*, *P. vivax*, *Plasmodium malariae*, and *Plasmodium ovale* followed. All positive and a random selection of 10% negative samples were assessed by independent double LM readings of asexual and sexual stages and parasite densities calculated. No discrepancy in parasite densities of above 30% occurred. Parasites were counted per approximately 500 leukocytes and densities were inferred assuming 8000 leukocytes per microlitre blood.

### Descriptive analyses

Villages were classified based on the forest cover in a 750 m radius around the households, computed from the land cover analysis in [28] (Additional file 1: Figure S1). Villages with  $\geq 50\%$  of households with  $\geq 10\%$  forest cover in their vicinity were considered “inside the forest”, with  $\geq 30\%$  of households with  $\geq 5\%$  forest cover as “at the forest fringe”, or “outside the forest” otherwise. Because of very low sample sizes, the two small, neighbouring villages Beng (11 individuals) and Gaty (95 individuals) were analysed as one. Population prevalence was estimated based on post-sampling weights assigned to each participant according to their representation by village, gender, and 10-year age bins compared to the census population (raw numbers of positive survey samples accompany the estimates in brackets). Categorical covariates were compared using the chi-squared test or the Fisher's exact test when low strata sizes required it.

### Risk factor analyses

The association of covariates with infection by *P. vivax*, *P. falciparum*, or all four species was assessed by mixed-effects logistic regression with random intercepts per household and village. Those covariates that were statistically significantly associated with *Plasmodium* infection at two-tailed  $\alpha=5\%$  in univariate regressions were included in the multivariate model. The villages' proximity to the forest, work-unrelated overnight travels (incl. to forest sites), and work in the deep forest were considered part of the multivariate model a priori in order to assess the association of forest exposure with risk of infection. Having slept outdoors or under a bed net last night were kept as part of the model based on causal reasoning or prior knowledge due to their association with infection risk in previous publications, *e.g.* [11]. Gender and age were retained in the model as proxies for risk-related behaviour and thus potential confounders. The other covariates comprised potential proxies for socio-economic status or further exposure variables and their subset significantly associated with infection risk was assessed by backwards variable selection. Akaike Information Criterion (AIC) was used to assess model fit. If collinearities and interactions were identified, covariates were retained comparing fit of the respective models by AIC. Statistical significance in the multivariate regressions was calculated per covariate by likelihood ratio tests. Spatial hotspots were identified by a purely spatial scan statistic via a discrete Poisson model with maximally a third of the population in a scanning window, adjusted for all covariates of the final multivariate model except for the villages' forest proximity [29].

**Software**

All questionnaires were applied on tablets using the REDCap (Research Electronic Data Capture) software hosted at Institut Pasteur in Paris [30, 31]. Data quality control as well as all descriptive and analytical statistics were performed in R 3.6.3 [32]. The SaTScan™ software version 9.6 was used for the spatial scan statistic [33].

**Results**

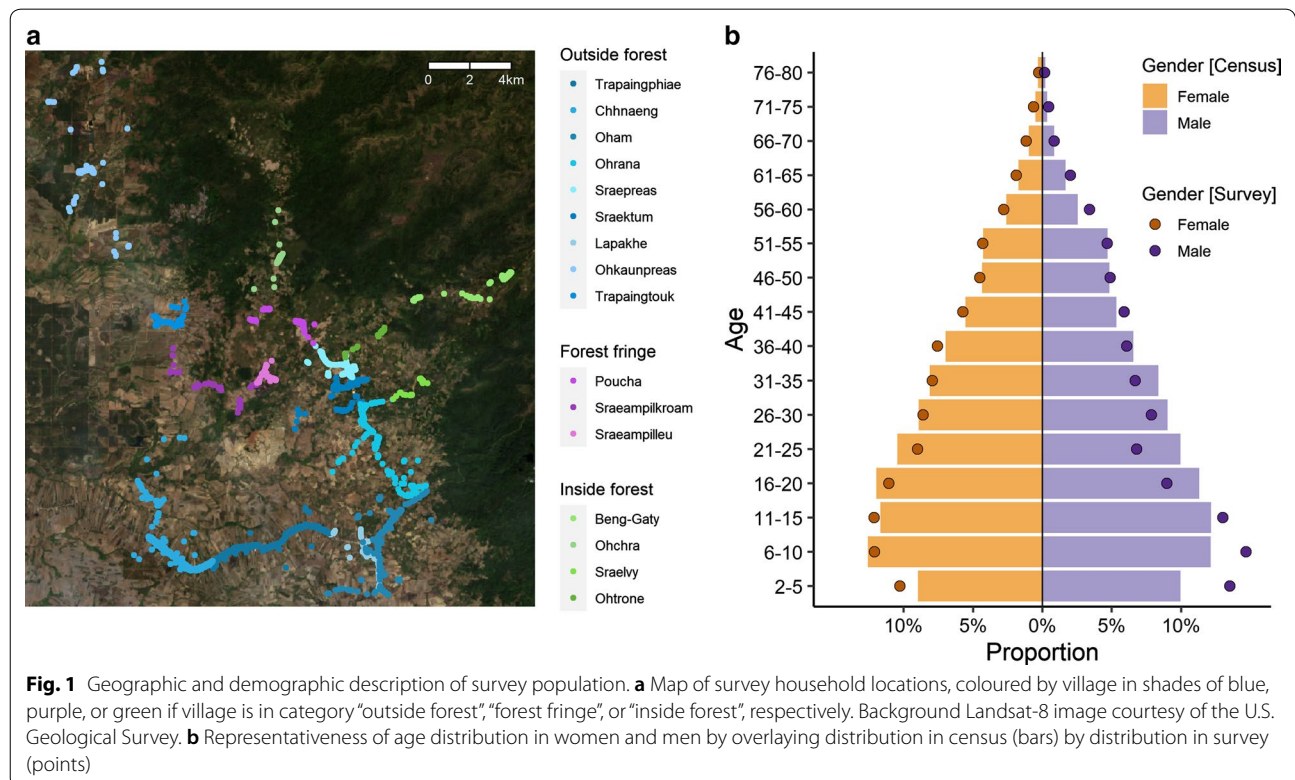
**Census, survey representativeness and survey population**

From among the 10,053 individuals in 2351 households identified in the census, the survey recruited 4200 participants from 1147 households and oversampled smaller villages on average (Additional file 1: Table ST1). A map of the survey households and the categorization of villages by proximity to the forest is shown in Fig. 1a. Mean age was 26 years in both the census and the survey and with 51% (5135/10,053) and 53% (2231/4200) women, respectively, women were slightly oversampled in the survey. In men, ages of 16 to 40 years were mildly underrepresented (Fig. 1b). The main income source for the survey households was by large majority farming (89.8%, 1030/1147). In terms of mobility, three-quarters (75.9%, 3188/4200) of the survey participants reported work-unrelated trips to forest or field sites in the last month (8.5%, 358/4200) or any work trip in the last two

months (75.8%, 3184/4200). By vast majority, these trips were short and frequent: In only 4.4% (140/3188) of the instances, stays for longer than a week were reported and among those who reported any work trip, 89.4% (2847/3184) went for work at least once a week.

**Plasmodium vivax predominated over other human malaria parasites**

Infection by *Plasmodium* parasite was detected by PCR in 8.3% (349/4200) of participants. The proportion of samples positive for *P. vivax* was 6.4% (268/4200) compared to 3.0% (125/4200) for *P. falciparum* ( $p < 0.001$ , Table 1). *Plasmodium vivax* was found in 77% (268/349) of all infections compared to 36% (125/349) for *P. falciparum*. Four samples were positive for *P. malariae* mono-infections and none for *P. ovale*. Speciation by PCR was unsuccessful in 4 samples which were analysed as negative. Extrapolation to the entire Kaev Seima population yielded an estimated prevalence of 8.9% for *Plasmodium* infection, 6.8% for *P. vivax*, and 3.3% for *P. falciparum*. Estimated prevalence was highly heterogeneous across the villages, ranging from 0.6% (3/594) to 36.3% (54/152) and from 0% (0/594) to 25.1% (27/106) for *P. vivax* and *P. falciparum*, respectively (both  $p < 0.001$ , Table 1). Village-level prevalence of *P. vivax* infection was on average higher in villages inside the forest compared with those



**Table 1** Prevalence of PCR-detected infections

	N	PCR positivity			
		<i>Plasmodium</i>	<i>P. vivax</i>	<i>P. falciparum</i>	Co-infections
Proportion (n) of survey samples	4200	8.3% (349)	6.4% (268)	3.0% (125)	1.1% (48)
Extrapolated population prevalence	10,053	8.9%	6.8%	3.3%	1.3%
Extrapolated prevalence per village					
<i>Outside forest</i>					
Trapaingphiae	594	0.6% (3)	0.6% (3)	0% (0)	0% (0)
Chhnaeng	550	2.7% (12)	1.9% (8)	0.9% (4)	0.3% (1)
Oham	333	8.0% (20)	6.2% (16)	2.9% (7)	1.1% (3)
Ohrana	470	5.7% (25)	4.2% (18)	1.8% (8)	0.2% (1)
Sraepreas	228	6.5% (13)	5.6% (11)	1.7% (3)	0.8% (1)
Sraektum	296	13.1% (36)	8.6% (24)	4.8% (13)	0.8% (2)
Lapakhe	71	10.6% (4)	6.7% (3)	7.8% (2)	3.9% (1)
Ohkaunpreas	317	6.9% (21)	5.9% (18)	1.7% (5)	0.7% (2)
Trapaingtouk	201	1.9% (3)	1.2% (2)	0.6% (1)	0% (0)
<i>Forest fringe</i>					
Poucha	239	9.7% (24)	6.7% (17)	4.3% (10)	1.4% (3)
Sraepilkroam	218	11.0% (23)	7.2% (15)	3.8% (8)	0.6% (1)
Sraepilleu	168	10.6% (17)	8.2% (13)	2.4% (4)	0% (0)
<i>Inside forest</i>					
Beng-Gaty	106	37.9% (40)	30.9% (32)	25.1% (27)	18.1% (19)
Ohchra	152	40.4% (60)	36.3% (54)	10.0% (15)	5.9% (9)
Sraelyy	154	18.7% (28)	15.5% (23)	3.9% (6)	1.3% (2)
Ohtrone	103	19.7% (20)	10.4% (11)	12.4% (12)	3.1% (3)

As proportions of positive blood samples or as census population-level estimates by extrapolation via post-sampling weights (raw numbers of positive samples in brackets). 4 positive *P. malariae* samples omitted

at the forest fringe and outside the forest (medians 23.2, 7.2, 5.6% respectively, Fig. 2; similarly for *P. falciparum* in Additional file 1: Fig. S2).

#### ***Plasmodium vivax* infections were least detectable by the health care system**

Of all PCR-detected infections, 83.2% (223/268) were sub-microscopic for *P. vivax* compared to 74.4% (93/125) for *P. falciparum* ( $p \approx 0.056$ , Table 2). *Plasmodium vivax* infections coincided less often with reported or measured symptoms at the interview than those by *P. falciparum*, with 6.7% (18/268) and 16.8% (21/125), respectively ( $p < 0.01$ ). Only 2.2% (6/268) of all *P. vivax* infections were symptomatic and also positive by RDT or LM (*i.e.* detectable through the Cambodian health care system), less often than for *P. falciparum* (7.2%, 9/125,  $p < 0.05$ ). Asexual parasite stages were detected in all LM<sup>+</sup> samples for *P. vivax* (37/37) and in most (81.5%, 22/27) for *P. falciparum* (Table 3). In LM<sup>+</sup> samples, the geometric mean parasite densities were 149.7 parasites/ $\mu$ L and 431.3 parasites/ $\mu$ L (*t*-test:  $p \approx 0.07$ ), respectively. While gametocytes were found in only one (2.7%, 1/37) *P. vivax* LM<sup>+</sup> sample, more than a third (37.0%, 10/27) of LM<sup>+</sup> samples for *P.*

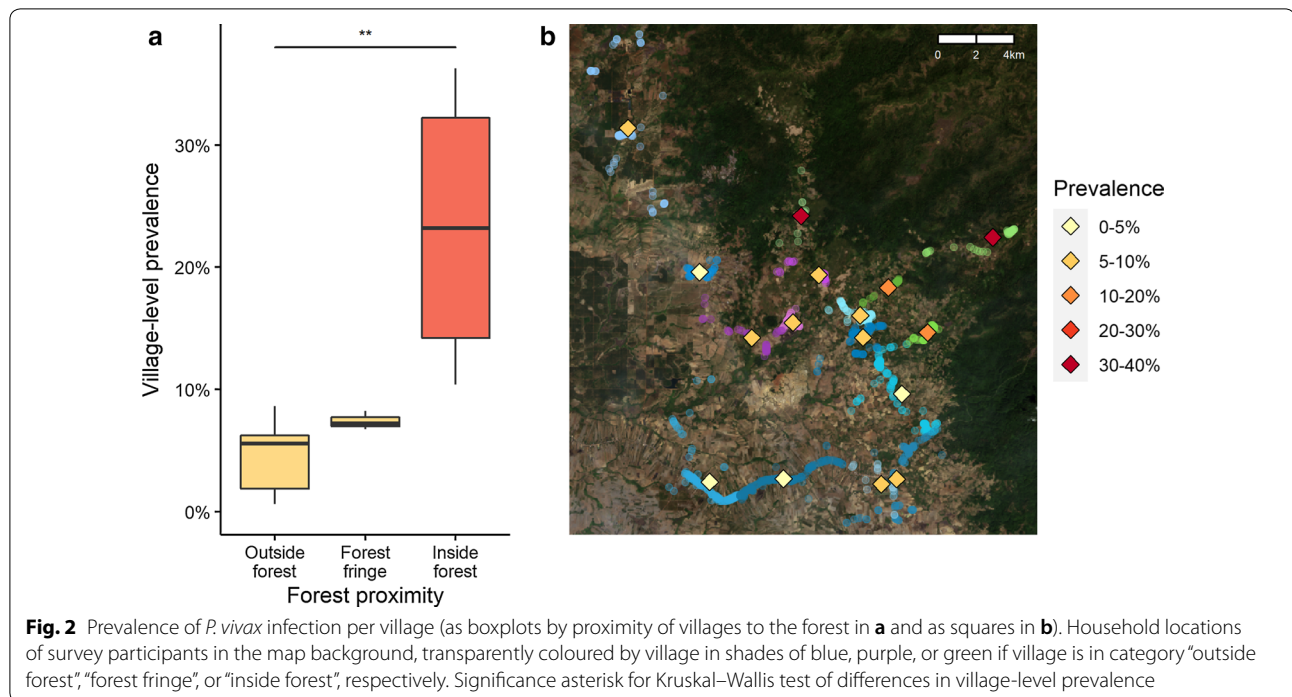
*falciparum* were gametocyte-positive with a geometric mean of 51.1 gametocytes/ $\mu$ L.

#### **Prevalence was highest in men of working age**

Estimated prevalence was more than twice as high in men as in women, *i.e.* 10.4% (188/1969) vs. 3.6% (80/2231) for *P. vivax* ( $p < 0.001$ ), 4.8% (83/1969) vs. 1.9% (42/2231) for *P. falciparum* ( $p < 0.001$ ), and 13.3% (239/1969) vs. 5.0% (110/2231) regardless of species ( $p < 0.001$ ). The patterns across age were similarly heterogeneous for both species (Additional file 1: Figure S3). While there was no difference in genus-wide prevalence across age in women, men showed an elevated risk at working age ( $p < 0.001$ ), regardless of the proximity of the village to the forest (Fig. 3). A fitted interaction term of gender and age was significant in the strata of villages outside the forest ( $p < 0.001$ ), but not across those villages at the forest fringe or inside the forest.

#### **Risk profiles for *P. vivax* and *P. falciparum* infections were similar**

Risk of infection was associated with individual covariates for both *P. vivax* and *P. falciparum* in a highly similar



**Table 2 Detectability of infections through the public health care system**

Measure	PCR positivity	
	<i>P. vivax</i>	<i>P. falciparum</i>
LM <sup>+</sup>	16.8% (45/268)	25.6% (32/125)
Symptoms at interview	6.7% (18/268)	16.8% (21/125)
Symptoms & LM <sup>+</sup>	2.2% (6/268)	4.8% (6/125)
Symptoms & RDT <sup>+</sup>	0.4% (1*/268)	4.0% (5/125)
Symptoms & RDT/LM <sup>+</sup>	2.2% (6/268)	7.2% (9/125)

Proportion (N) of PCR-positive samples that were also detectable by LM, inspection of symptoms, or by testing of symptomatic individuals with LM or RDTs

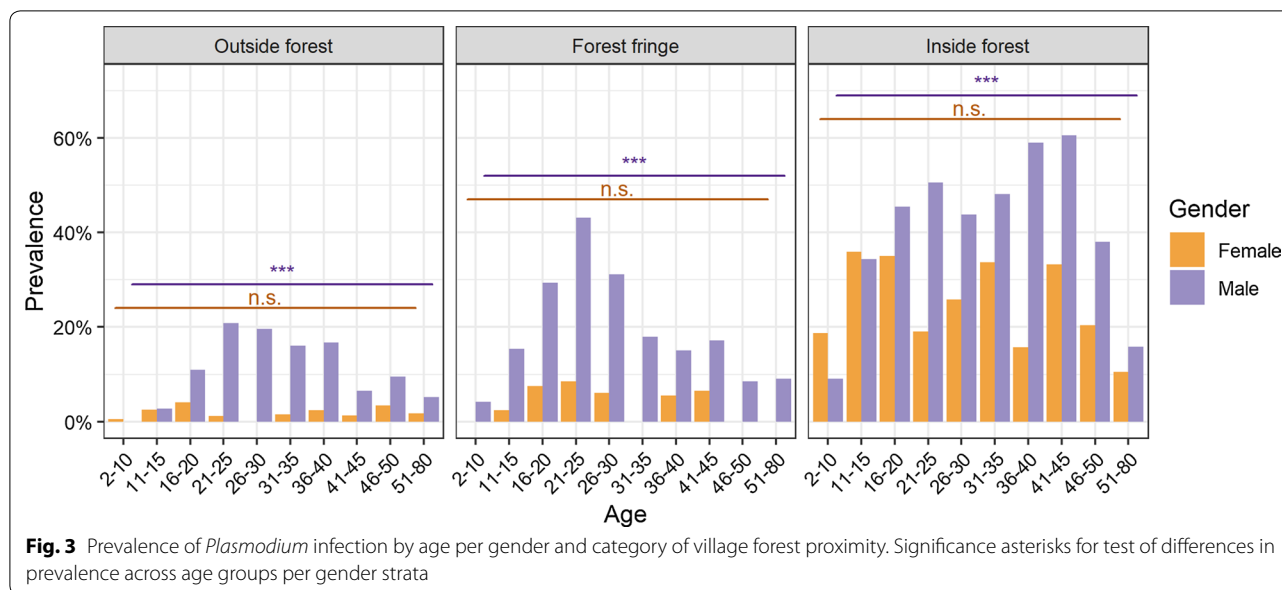
\*Actually RDT<sup>+</sup> for *P. falciparum* in a co-infection with *P. vivax* as by PCR

fashion (Table 4 for behavioural variables, full list of variables with odds ratios in Additional file 1: Table ST2). For both species, prevalence was associated with work-unrelated overnight travels and highest if those occurred to forest sites (22.4%, 60/268, and 14.9%, 40/268, for *P. vivax* and *P. falciparum*, respectively) and lowest for urban destinations (1.7%, 1/59, and 0%, 0/59, for both  $p < 0.001$ ). Infections of both species were also more prevalent among those who reported work trips to sites in the nearby and deep forest (assessed separately). In particular, a significantly higher prevalence was observed in participants who reported work trips into the deep forest (25.5%, 37/145, and 18.6%, 27/145) compared to those who did not (5.7%, 231/4055, and 2.4%, 98/4055,

**Table 3 Densities of asexual and sexual stages in LM<sup>+</sup> samples**

	LM positivity	
	<i>P. vivax</i>	<i>P. falciparum</i>
<i>Asexual parasitaemia</i>		
Proportion (N) of LM <sup>+</sup> samples with detected asexual parasites	100% (37/37)	81.5% (22/27)
Geometric mean [ $\mu$ l blood]	149.7	431.3
Range [ $\mu$ l blood]	15.4–22,674.7	14.3–41,724.6
<i>Gametocytaemia</i>		
Proportion (N) of LM <sup>+</sup> samples with gametocytes	2.7% (1/37)	37.0% (10/27)
Geometric mean [ $\mu$ l blood]	31.9	51.1
Range [ $\mu$ l blood]	31.9–31.9	15.6–175.0

Geometric mean and range always given from among samples with non-zero densities



for *P. vivax* and *P. falciparum*, respectively,  $p < 0.001$ ). Once clustering at household and village-level was taken into account in univariate mixed-effects logistic regression, work in the nearby forest was no longer significantly associated with risk of infection though (Additional file 1: Table ST2). There were fewer infections among those who reported the use of standard protection measures such as sleeping under a bed net (6.1%, 236/3880, and 2.7%, 104/3880, vs. 10.0%, 32/320, and 6.6%, 21/320, if no net was used,  $p < 0.01$  and  $p < 0.001$  for *P. vivax* and *P. falciparum*, respectively). A higher risk in men, at working age, in villages at the forest fringe or inside the forest, and with indicators of lower socio-economic status was also found similarly for both species (Additional file 1: Table ST2).

**Working in and travelling to the forest were strong risk factors of infection**

Behavioural covariates related to activities in the forest were statistically significant in the multivariate model of *Plasmodium* infection (Table 5). Having travelled to forest sites (nearby or deep forest) or having worked in the deep forest independently increased the odds of infection two to three-fold (adjusted odds ratio, aOR, 2.17,  $p < 0.01$  and aOR 2.88,  $p < 0.001$ , respectively). Risk of infection was not significantly attributed to the other behavioural covariates that were included in the model a priori, namely having slept outdoors (aOR 1.99,  $p \approx 0.08$ ) and under a bed net (aOR 0.99,  $p \approx 0.96$ ). Risk of infection was increased in males (aOR 3.06,  $p < 0.001$ ) and working age (aOR 7.84 in 21–25 years old compared to children,  $p < 0.001$ ). A fitted interaction term of gender and age

improved the model (AIC 1786 vs. 1802 without interaction,  $p < 0.001$ , Additional file 1: Table ST3). Other covariates linked higher socio-economic status with lower odds of infection, such as living in a house with a roof built of relatively high-quality material (aOR 0.50,  $p < 0.01$ ). The covariate on recent information on malaria via TV (aOR 0.37,  $p < 0.01$ ) most likely also acts as a proxy for higher socio-economic status, i.e. being able to afford a TV in the first place.

**Descriptive characterization of the risk factors forest work and forest travels**

Work-unrelated overnight travels to forest sites was a predominantly male domain (reported by 10.7%, 211/1969, of the men vs. 2.6%, 57/2231, of the women,  $p < 0.001$ ) and particularly frequent in male adolescents (with 24.3%, 70/289, highest in 21–30 years old males,  $p < 0.001$ ). Forest work was independently assessed for sites of the nearby and deep forest. Only for the latter, a statistically significant association with risk was observed. As for travels, they were reported more frequently by men than by women (6.9%, 135/1969, vs. 0.4%, 10/2231,  $p < 0.001$ ) and most often by 21–30 years old males (17.6%, 51/289,  $p < 0.001$ ). However, working in the deep forest occurred in both men aged between 8 and 60 years and in 8–43 years old women. Forest travels were reported in 3–62 years old men and 3–63 years old women. Both kind of trips occur usually frequently and of short duration. In 81% (217/268) of the times, the travels to forest sites lasted less than a week. Work trips into the deep forest occurred in 75% (108/145) of the times at least weekly. When going for work several times



**Table 4 Univariate association of infection risk and behavioural covariates**

Covariate	N	<i>P. vivax</i>		<i>P. falciparum</i>	
		Prevalence	p-value	Prevalence	p-value
<i>Work-unrelated travels overnight in the last month to...</i>					
None	3465	4.9% (171)	< 0.001	2.0% (70)	< 0.001
Field sites	90	3.3% (3)		4.4% (4)	
Forest sites	268	22.4% (60)		14.9% (40)	
A village	177	13.6% (24)		5.6% (10)	
A city	59	1.7% (1)		0% (0)	
Unspecified	123	7.3% (9)		0.8% (1)	
<i>Work in the last two months in...</i>					
...deep forest					
No	4055	5.7% (231)	< 0.001	2.4% (98)	< 0.001
Yes	145	25.5% (37)		18.6% (27)	
...cassava field					
No	2115	5.6% (118)	< 0.05	2.9% (62)	n.s.
Yes	2085	7.2% (150)		3.0% (63)	
No work					
No	3186	7.9% (253)	< 0.001	3.7% (119)	< 0.001
Yes	1014	1.5% (15)		0.6% (6)	
<i>Slept outdoors last night</i>					
Indoors	4124	6.2% (256)	< 0.01	2.9% (118)	< 0.01
Outdoors	76	15.8% (12)		9.2% (7)	
<i>Sprays repellent usually at bedtime</i>					
No	3668	6.7% (247)	< 0.05	3.2% (116)	< 0.10
Yes	524	4.0% (21)		1.7% (9)	
<i>Slept under net last night</i>					
No	320	10.0% (32)	< 0.01	6.6% (21)	< 0.001
Yes	3880	6.1% (236)		2.7% (104)	

Prevalence (n) of PCR-positivity across the strata of those behavioural variables that were statistically significant in univariate logistic regression. "n.s.": Not significant

a month or once a month, the reported duration was also most often less than a week (83%, 15/18, and 53%, 8/15, of the times, respectively). A net was used during overnight work trips into the forest in only 25% (28/113) of the instances. While equally few participants from villages outside the forest, at the forest fringe, and inside the forest reported work in the deep forest (3.4%, 105/3060, 2.9%, 18/625, and 4.3%, 22/515, respectively,  $p \approx 0.44$ ), travelling to forest sites was reported more often in forest fringe and forest villages (8.6%, 54/625, and 11.5%, 59/515, respectively, compared to 5.1%, 155/3060, outside the forest,  $p < 0.001$ ).

**Residing in a village inside the forest was an independent spatial risk factor**

Living in a village inside the forest remained associated with risk of infection when adjusting for the significant

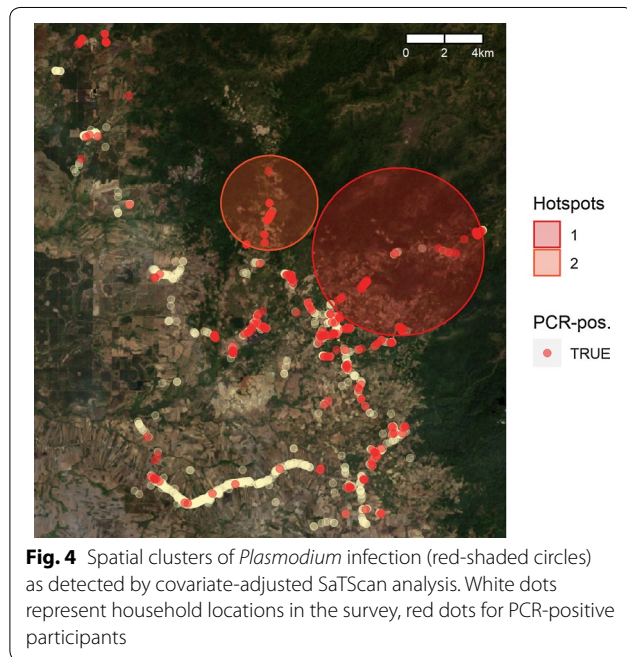
**Table 5 Risk factors after multivariate mixed-effects logistic regression for *Plasmodium* infection as detected by PCR**

Covariate	N	aOR	95% CI	p
Gender				
Female	2231	Reference		< 0.001
Male	1969	3.06	[2.26–4.13]	
Age [years]				
2–10	1054	Reference		< 0.001
11–15	527	4.51	[2.52–8.06]	
16–20	424	7.47	[4.14–13.47]	
21–25	335	7.84	[4.20–14.63]	
26–30	347	8.08	[4.36–14.94]	
31–35	309	7.71	[4.05–14.70]	
36–40	289	7.15	[3.76–13.58]	
41–45	244	5.33	[2.67–10.65]	
46–50	197	4.16	[1.92–8.99]	
51–80	474	2.68	[1.36–5.29]	
Forest proximity of village				
Outside forest	3060	Reference		< 0.001
Forest fringe	625	2.14	[1.02–4.52]	
Inside forest	515	12.47	[6.29–24.71]	
Material of roof				
Grass/leaves	81	0.96	[0.26–3.55]	< 0.01
Tent	79	3.43	[1.36–8.64]	
Corrugated iron	3301	Reference		
Wood planks/cement/tiles	739	0.50	[0.30–0.84]	
Household owns a toilet				
No	2383	Reference		< 0.05
Yes	1816	0.69	[0.48–1.00]	
Household owns buffaloes				
No	3977	Reference		< 0.01
Yes	222	2.11	[1.21–3.69]	
Household head had received information on malaria via TV in the past 3 months				
No	3784	Reference		< 0.01
Yes	415	0.37	[0.16–0.81]	
Insecticides had been sprayed inside the house in the past year				
No	2935	Reference		< 0.01
Yes	1264	0.62	[0.43–0.89]	
Slept outdoors last night				
No	4124	Reference		0.08
Yes	76	1.99	[0.93–4.25]	
Slept under net last night				
No	320	Reference		0.96
Yes	3880	0.99	[0.61–1.59]	

demographic, socio-economic, and behavioural covariates (aOR 12.47,  $p < 0.001$ , Table 5). Households from all four villages inside the forest also formed hotspots of *Plasmodium* infection, i.e. purely spatial clusters of elevated risk of infection that cannot be explained by

**Table 5 (continued)**

Covariate	N	aOR	95% CI	p
Work-unrelated travels overnight in the last month to...				
None	3465	Reference		< 0.01
Field sites	90	0.63	[0.25–1.59]	
Forest sites	268	2.17	[1.41–3.35]	
A village	177	1.28	[0.75–2.17]	
A city	59	0.27	[0.03–2.13]	
Unspecified	123	1.23	[0.56–2.74]	
Work in the last two months in...				
...deep forest				
No	4055	Reference		< 0.001
Yes	145	2.88	[1.69–4.93]	
...cassava field				
No	2115	Reference		< 0.05
Yes	2085	1.37	[1.01–1.85]	



the other covariates (Fig. 4, Additional file 1: Tables ST4–ST5).

## Discussion

This is the first detailed study on the prevalence of *Plasmodium* infection and associated risk factors on population-level in Mondulakiri province in Cambodia. The overall prevalence of *Plasmodium* infection is consistent with that observed in the neighbouring province of Ratanakiri [10, 11, 22] or other endemic provinces in the West of the country [16].

*Plasmodium falciparum* has long been the predominant species countrywide [9, 34]. However, following a scale-up in the VMW programmes, a steady decrease in reported *P. falciparum* cases was observed in 2009–2011, while numbers of reported *P. vivax* cases increased [24]. In recent years, cases of both species were reported at an almost equal share [4] and in 2018 *P. vivax* accounted for approximately three quarters of reported cases [2]. That *P. vivax* started to predominate over *P. falciparum* in Cambodia even earlier is suggested by other studies in high-incidence provinces where molecular and serological diagnosis identified a higher prevalence of *P. vivax* than *P. falciparum* infections [11, 12, 15–19], consistent with the presented study in Mondulakiri.

7.2% of the *P. falciparum* infections could be identified by symptoms and a positive result by LM or RDTs, *i.e.* the diagnostics used by the health system, compared to only 2.2% for *P. vivax*. Low sensitivity of RDTs for *P. vivax* is a commonly reported problem [12, 35–38]. In addition, higher proportions of asymptomatic infections for *P. vivax* are regularly observed as the relapses can lead to higher levels of immunity and thus overall lower parasite densities [39, 40]. Data from studies using membrane feeding assays suggest that the densities of asexual stages and gametocytes in the asymptomatic, LM-positive samples in this survey are infectious to mosquitoes [14, 41–45]. It can thus not be ruled out that these subclinical infections contribute to the ongoing transmission in this area.

More asymptomatic infections, lower diagnostic sensitivity for *P. vivax*, high transmissibility, and the lack of radical cure by primaquine all might add up as possible explanations why the control efforts have been less successful against *P. vivax* than against *P. falciparum*. In order to address this high burden of *P. vivax*, Cambodia is now piloting the use of primaquine against *P. vivax* infections in four provinces. While an essential step, the effect of radical cure will be limited without point-of-care diagnostics that are more sensitive for *P. vivax* infections and ideally a test allowing the detection of hypnozoite carriage (irrespective of blood stage parasitaemia). New advances in the use of multiple antigens to detect antibodies as serological markers for recent exposure and thus for potential hypnozoite carriers are promising in this respect [46].

With as many as 40% of the inhabitants infected in the highest-prevalence village in this survey, it may be necessary to complement the current malaria control efforts by active or reactive case detection schemes [47, 48] to reduce the infected (and potentially infectious) reservoir and eventually reach the goal of malaria elimination. Targeting such resource-intensive interventions to high-risk groups will render them significantly more cost-efficient.

The similarity of the risk profiles for both *P. falciparum* and *P. vivax* presented here is encouraging as it suggests that such targeted interventions would appropriately address both of the two species.

This study emphasizes the predominant role of forest malaria transmission in explaining elevated risk of infection in remote rural areas of Southeast Asia. Previous cross-sectional surveys have also identified an association of infection risk with either work in the forest [15, 17, 49] or time spent therein [16, 50]. However, this study is unique in retaining them both as statistically significant risk factors in one multivariate model while also adjusting for gender, age, socio-economic proxies, and other behavioural covariates. While work in and travels into the forest are most frequent in adolescent and adult men who are usually considered the forest-related risk group, this behaviour also occurred in women and from children to participants in their sixties and thus actually extends to a much broader range of the population.

On the relatively small spatial scale of 17 villages in an area of  $22 \times 26 \text{ km}^2$ , the prevalence ranged from less than 1% to above 40% in the dry season. The trend towards higher prevalence in villages at the forest fringe and inside the forest compared to those outside the forest is in line with the widely accepted notion for forest-related mosquito abundance and exposure in Cambodia. Other studies have also identified a higher prevalence in villages at close proximity to the forest [21, 49, 50]. It is apparent that the association of risk with living near or inside the forest was retained in this study even if adjusting for demographic, socio-economic, occupational, and other behavioural differences.

Not more than 60% of infections could be attributed to the strong behavioural, forest-related risk factors. In villages outside the forest or at the forest fringe, most infections indeed occurred in working age men. This predominance of this occupational risk group was much lower in forest fringe villages and entirely lost in villages situated inside the forest. Taking gender and age as proxies for risk-related behaviour and occupational activities, this could indicate that in villages outside the forest, risk can be explained almost completely by such occupational exposure. By contrast, for villages within the forest, the more homogeneous presence of infection across all age groups in both males and females indicates additional exposure inside the village. This is in line with other studies in which household-level risk factors in forested areas were important to explain infection risk [10] or infections clustered in households over 2 years [51] in Ratanakiri, the other Eastern province of Cambodia.

The current WHO report on malaria eradication calls for subnational stratification of intervention programmes [52, 53]. This study suggests that such a stratification of interventions could happen along a gradient of villages inside or outside the forest. Population-oriented interventions such as targeted mass drug administration or test and treat programmes with sensitive molecular or serological diagnostics [46, 54] (together with their costs and risk of overtreatment) could be justified in forest villages. By contrast, approaches targeted at risk groups only may be effective at lower costs for the NMCP and the local population in residential settings outside of the forest.

## Conclusion

Despite a substantial reduction in malaria burden in Cambodia over the last decades, this study demonstrates that pockets of high malaria prevalence persist in the country. Given that the vast majority of infections were asymptomatic, the study strengthens the argument to enhance malaria elimination efforts by measures of (re-)active detection of also asymptomatic infections. *Plasmodium vivax* infections were detected at a higher prevalence than *P. falciparum* infections, more often asymptomatic, and less detectable by RDT and LM. Consequently, novel tools for the identification of hypnozoite carriers could play a key role in the presented setting as well as the roll-out of routine radical cure treatment. The study corroborates the notion of forest malaria in the Greater Mekong Subregion by demonstrating the independent association of travels to the forest, of forest work, and of living in close proximity to forest with malaria infection risk. However, the study also demonstrates that infection risk is less confined to forest-goers in sub-populations that already live in forested areas and suggests within-village transmission therein. A focus of interventions solely on forest-goers could thus be insufficient to reach the goal of nation-wide malaria elimination in due time. In the light of the presented results, interventions could be targeted at whole villages inside the forest, and targeted at stratified risk groups for villages outside the forest.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s12936-020-03482-4>.

**Additional file 1.** Additional tables ST1–ST5 and additional figures S1–S3.

### Abbreviations

ACT: Artemisinin-based combination therapy; AIC: Akaike Information Criterion; aOR: Adjusted odds ratio; G6PD: Glucose-6-phosphate dehydrogenase; GPS: Global positioning system; K<sup>+</sup>EDTA: Potassium ethylenediaminetetraacetic acid; LLIN: Long-lasting insecticidal net; LM: Light microscopy; MMW: Mobile malaria worker; N: Sample size; NMCP: National malaria control programme; PCR: Polymerase chain reaction; RDT: Rapid diagnostic test; VMW: Village malaria worker; WHO: World Health Organization.

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### Authors' contributions

AV, LR, BW, and IM conceived and designed the study and were overall responsible for the study. SK, AV, and BW implemented and managed the data collection, with support by SG, NK, and DL, and supervised the sample processing. SK conducted the data collection, supported by data management by MS and TO. TO, MS, SK, AV, LR, and IM designed the questionnaires. MW contributed to the statistical analysis. AP contributed the land cover analysis. MS analysed the data and wrote the first draft of the manuscript. AV, MW, TO, BW, LR, and IM contributed to data interpretation and writing. All authors read and approved the final manuscript.

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### Availability of data and materials

The de-identified dataset analysed for this study is being made publicly available in ClinEpiDB repository, <https://clinepidb.org/ce/app/>. In the meantime, it is available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

This study was approved by the National Ethics Committee for Health Research, Ministry of Health, Kingdom of Cambodia (reference 239NECHR) and by the Institut Pasteur Institutional Review Board (reference 2017-03). Written informed consent to participate in the study was obtained from all participants (or their parent or legal guardian).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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**Chapter 4: The spatial signature of *Plasmodium vivax* and *P. falciparum* infections: Quantifying the clustering of infections in cross-sectional surveys and cohort studies**

1 The spatial signature of *Plasmodium vivax* and *P. falciparum*  
2 infections: Quantifying the clustering of infections in cross-  
3 sectional surveys and cohort studies

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## 26 Abstract

27 **Background:** Over the last decades, enormous successes have been achieved in reducing malaria  
28 burden globally. In Latin America, South East Asia, and the Western Pacific, many countries now  
29 pursue the goal of malaria elimination by 2030. It is widely acknowledged that *Plasmodium spp.*  
30 infections cluster spatially so that interventions need to be spatially informed, e.g. spatially targeted  
31 reactive case detection strategies. We introduce the spatial signature method as a tool to quantify  
32 the distance around an index infection within which other infections significantly cluster.

33 **Methods:** We considered data from cross-sectional surveys from Brazil, Thailand, Cambodia, and  
34 Solomon Islands, conducted between 2012 and 2018. Household locations were recorded by GPS and  
35 finger-prick blood samples from participants were tested for *Plasmodium* infection by PCR. We also  
36 included cohort studies from Brazil and Thailand with monthly sampling over a year from 2013 until  
37 2014. We calculated the prevalence of PCR-confirmed infections at increasing distance around index  
38 infections (and growing time intervals in the cohort studies). Statistical significance was defined as  
39 prevalence outside of a 95%-quantile interval of a bootstrap null distribution after random re-  
40 allocation of locations of infections.

41 **Results:** Prevalence of *Plasmodium vivax* and *P. falciparum* infections was elevated in close proximity  
42 around index infections and decreased with distance in most study sites, e.g. from 21.3% at 0 km to  
43 the global study prevalence of 6.4% for *P. vivax* in the Cambodian survey. In the cohort studies, the  
44 clustering decreased with longer time windows. The distance from index infections to a 50%  
45 reduction of prevalence ranged from 25 m to 3,175 m, tending to shorter distances at lower global  
46 study prevalence.

47 **Conclusions:** The spatial signatures of *P. vivax* and *P. falciparum* infections demonstrate spatial  
48 clustering across a diverse set of study sites, quantifying the distance within which the clustering  
49 occurs. The method offers a novel tool in malaria epidemiology, potentially informing reactive



50 intervention strategies regarding radius choices of operations around detected infections and thus  
51 strengthening malaria elimination endeavours.

## 52 Keywords

53 Malaria; *Plasmodium vivax*; *Plasmodium falciparum*; spatial epidemiology; spatial clustering;  
54 spatiotemporal clustering; spatial signature

## 55 Background

56 Globally, enormous successes have been achieved over the last decades in reducing the malaria  
57 burden (1). The main instruments were the distribution of long-lasting insecticide-treated nets and  
58 clinical case management after passive case detection. The strategies deployed so far have proven  
59 particularly successful in battling *P. falciparum* transmission. However, *P. vivax* causes relapses (2)  
60 and to a higher degree asymptomatic low-parasitaemia infections that escape passive case detection  
61 but contribute to transmission (3, 4). The effectiveness against *P. vivax* malaria has thus been less  
62 pronounced so that *P. vivax* has predominance over *P. falciparum* in many areas (1).

63 In Latin America, South East Asia, and the Western Pacific, the overall reduction of malaria burden in  
64 many countries has paved the way for the ambitious goal of malaria elimination by 2030 (1, 5). The  
65 necessity to incorporate active finding of infections in order to tackle the silent reservoir of on-going  
66 transmission is increasingly acknowledged (3, 4, 6-9). One strategy considered as part of current  
67 recommendations for malaria elimination are reactive interventions, i.e. active finding, testing, and  
68 treatment of other infections in a delimited area around passively detected malaria cases (10).  
69 However, the radius of the area in which to optimally implement interventions remains unclear.

70 Infection with human *Plasmodium spp.* through an *Anopheles spp.* mosquito bite depends on a  
71 complex interplay of factors of the pathogen, host, vector, and environment. Each of these factors,  
72 such as proximity to the vector's breeding grounds, bed net usage, *Anopheles* species composition,  
73 and host immunity, can vary geographically (11, 12). Geographical heterogeneity in malaria

74 infections appears across multiple spatial scales varying from between households (13-16) to within  
75 and between villages (9, 11). At low incidence, the possibility to identify single malaria transmission  
76 clusters has long been reported (17).

77 Reducing malaria transmission country-wide usually leads to a geographical fragmentation of areas  
78 where malaria risk remains high, further increasing spatial heterogeneity (18). Tailoring interventions  
79 to high-risk areas within these countries is at the core of recommended strategies for malaria  
80 elimination (10, 19). Obtaining a good understanding of the spatial dependence between malaria  
81 infections will help underpin spatially targeted control efforts.

82 To date, most efforts to characterise the spatial clustering of malaria have relied on comparing  
83 infection prevalence at community or household scales (11, 13). In addition, some studies have used  
84 space-time clustering statistics such as the *k*-nearest neighbour method for binary testing whether  
85 spatiotemporal clustering of cases occurred (17). The introduction of spatial scanning (20, 21) to  
86 malaria epidemiology further stimulated studies on spatial heterogeneity in disease or infection risk.  
87 This method scans the study area for (size-variable) circular areas with statistically significant  
88 clustering of infections ('hotspots'). Such hotspots were identified in diverse study areas in South  
89 America (22-26), Africa (27-39), and South East Asia (9, 40-44), for *P. falciparum* (25, 32, 40, 41), *P.*  
90 *vivax* (25, 26, 32, 40, 41), and *P. malariae* (40, 41). However, the method's effectiveness in  
91 forecasting hotspots remains unclear and it does not generalize spatial patterns between infections.

92 In this study, we introduce a global clustering statistic to malaria epidemiology, borrowing strongly  
93 from earlier development in the epidemiology of dengue and other pathogens (45, 46). The spatial  
94 signature quantifies both the magnitude of clustering of infections and the radius within which it is  
95 statistically significant, potentially informing reactive interventions as part of malaria elimination  
96 endeavours. We reveal spatial signatures of PCR-confirmed *P. vivax* and *P. falciparum* infections by  
97 assessing the pooled prevalence of infections around confirmed index infections in widening spatial  
98 and temporal windows. We demonstrate spatial and temporal clustering of infections in both cross-

99 sectional surveys and cohort studies from a diverse spectrum of study sites in Brazil (6, 47), Thailand  
100 (7, 8), Cambodia (9), and Solomon Islands (48).

## 101 **Methods**

### 102 **Cross-sectional malaria prevalence surveys**

103 We used data from 5 cross-sectional surveys from 4 countries, i.e. Brazil, Thailand, Cambodia, and  
104 Solomon Islands. The study design and basic descriptions are summarized in Table 1.

105 We included a cross-sectional survey in 17 villages in the high-incidence, rural province Mondulhiri, in  
106 North-Eastern Cambodia, in the dry season in December 2017 until April 2018 (as described in (9)).  
107 The 4,200 study participants were 2-79 years old and from randomly sampled households based on a  
108 census prior to the survey. We classified villages of residence into categories of proximity to the  
109 forest based on a forest cover remote sensing analysis (49). In this and all following included studies,  
110 we tested finger-prick blood samples for *P. vivax* and *P. falciparum* infections by real-time PCR and  
111 collected GPS locations of households.

112 Table 1 Design and basic summary of included cross-sectional surveys and cohort studies.

Study type	Country (reference)	Number of participants	Timing and duration	<i>P. vivax</i> PCR prevalence	<i>P. falciparum</i> PCR prevalence	Median age [range]	Gender [%]
Cross-sectional surveys (sampling once)	Cambodia (9)	4,200	Dec 2017-Apr 2018 (dry season)	6.4%	3.0%	22 years [2-79]	Female 53%
	Thailand (7)	4,309	Sep-Oct 2012 (low transmission season)	3.1%	0.9%	20 years [0-92]	Female 52%
	Brazil (6)	2,010 (Survey 1)	Nov 2012-Jan 2013 (rainy season)	4.3%	0.8%	23 years [0-100+]	Female 47%
		2,073 (Survey 2)	Aug-Sep 2013 (dry season)	3.4%	<0.1%	25 years [0-100+]	Female 48%
	Solomon Islands (48)	3,501	May-June 2012 (minimal seasonality)	13.4%	0.1%	18 years [1-100]	Female 53%
Cohort studies (monthly sampling)	Thailand (8)	999 (14 sampling visits)	May 2013-Jun 2014	1.7-4.2%*	0-1.3%*	23 years [1-82]	Female 54%
	Brazil (47)	1,274 (13 sampling visits)	Apr 2013-Mar 2014	2.5-6.5%*	0-1.0%*	25 years [0-100+]	Female 49%

113 \* Range of monthly prevalence. † Some individuals had reported ages greater than 100 years. In these cases, record keeping was considered not sufficiently  
 114 robust to provide an accurate estimate of age.

115

116 From Kanchanaburi and Ratchaburi provinces in western Thailand, we consider data from a cross-  
117 sectional survey in September-October 2012 (7). We enrolled 4,309 study participants aged 0-92  
118 years.

119 We added data from two cross-sectional surveys in a peri-urban part of Manaus, Amazonas State,  
120 Brazil from November 2012 until January 2013 (rainy season) and from August until September 2013  
121 (dry season), respectively (6). After all inhabitants had been invited to the study, 2,010 and 2,073  
122 participated in the two surveys, respectively (963 in both surveys).

123 From Solomon Islands, we used data from a cross-sectional survey in several villages across Ngella,  
124 Central Islands province from May until June 2012 (48). From a representative household-based  
125 sample, 3,501 individuals agreed to participate.

#### 126 Longitudinal cohort studies

127 In addition to the cross-sectional surveys, we used data from two longitudinal cohort studies with  
128 similar design: Monthly active follow-up with finger-prick blood-sampling for *P. vivax* and *P.*  
129 *falciparum* real-time PCR testing and collection of household locations by GPS at baseline. One study  
130 was conducted in two villages in Kanchanaburi and Ratchaburi provinces, Thailand, respectively, in  
131 May 2013 until June 2014, enrolling 999 participants (Table 1) (8). The other study took place in the  
132 peri-urban part of Manaus, Brazil, from April 2013 until March 2014 with 1,274 enrolled participants  
133 (47).

#### 134 Spatial signature of malaria infection

135 In order to characterise the spatial dependence between infections in both the cross-sectional  
136 surveys and for each follow-up period in the longitudinal studies, we calculated the average  
137 prevalence  $\pi(d)$  of PCR-positive infections within a distance  $d$  of PCR-positive “index” infections. To  
138 do this, we identified the study participants that lived within distance  $d$  of each PCR-positive  
139 participant, and calculated the proportion that were also PCR-positive. We varied  $d$  in incremental  
140 steps by 1 km for  $\pi$  across the entire study region; and by 50 m for  $\pi$  within 1 km.

141 We compared the observed prevalence with that expected under a null distribution where no spatial  
142 dependence exists. We generated a bootstrap null distribution of  $\pi$  after 10,000 random re-  
143 allocations of infection locations and define statistical significance outside of the null's 95<sup>th</sup> percentile  
144 interval.

145 For the cohort studies, we calculated the spatial signature for *P. vivax* infections as above, pooling  
146 across the monthly study visits, i.e. considering only index-neighbour pairs in the same study month.  
147 If multiple monthly PCR results were positive for an individual, we consider only the first, "incident"  
148 positive result.

149 We provide the underlying distribution of pairwise distances between the study participants per  
150 study site as well as the underlying distribution of pairwise distances between infections and all study  
151 participants in the Supplementary Figures (S.1.1a-S1.7).

## 152 Spatiotemporal signature of malaria infection

153 In the two longitudinal cohort studies, we also explored the extent of spatiotemporal dependence.

154 We calculated the period prevalence  $\rho(d, t)$  of PCR-positive neighbouring infections within a  
155 distance  $d$  and within a time window  $t$  of all incident PCR-positive index infections for *P. vivax* as the  
156 ratio of the number of pairs of index and neighbouring incident infections within  $d$  and  $t$  and the  
157 number of pairs of study participants within  $d$  (steps by 1 km for  $\rho$  across the entire study region; by  
158 50 m for  $\rho$  within 1 km). A study participant was considered an incident infection at the first study  
159 visit with a PCR-positive blood sample. We generated a bootstrap null distribution of  $\rho$  after 1,000  
160 random re-allocations of locations of infection time series.

161 From the cohort study in Thailand, PCR-positive blood samples for *P. vivax* were genotyped as  
162 described in (8). We calculated spatiotemporal signatures as described above but respecting only *P.*  
163 *vivax* infection pairs with a matching genotype.

## 164 Comparative analysis of distance to 50% reduction in prevalence

165 From each spatial signature across the cross-sectional surveys and cohort studies, we drew the  
166 distance within which the prevalence around index infection has fallen by 50% (between the  
167 prevalence at 0 m and the study's global prevalence). The distance was approximated as the mean of  
168 the pair of 50 m-increments of distance  $d$  within which the spatial signature falls below the 50% of  
169 prevalence.

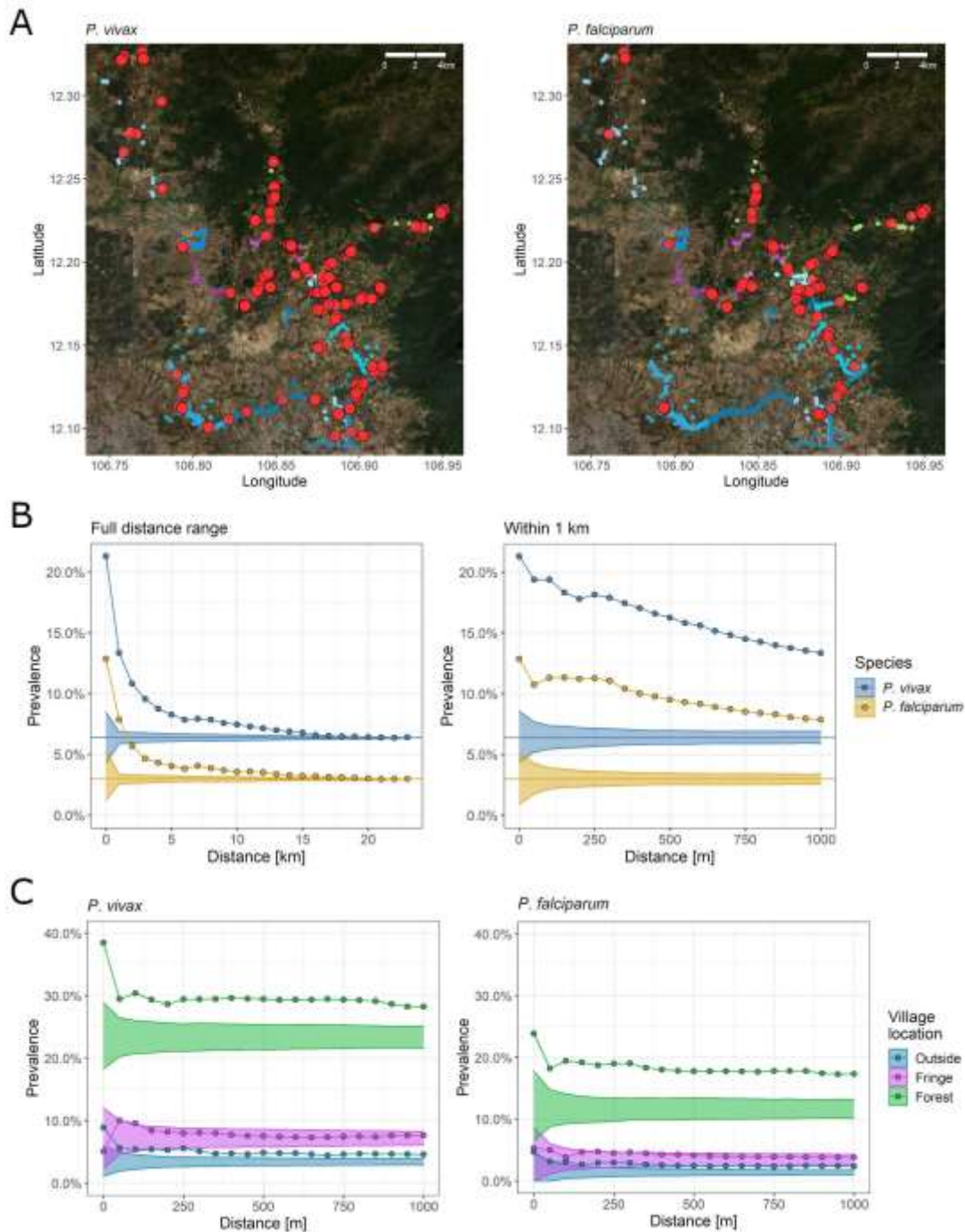
## 170 Software

171 We described, analysed, and visualised the data in R 4.1.2 (50). We created the maps with the  
172 ggmaps package in R and background Landsat-8 image courtesy of the U.S. Geological Survey.

## 173 Results

### 174 Spatial signatures from cross-sectional surveys

175 In the Cambodian cross-sectional survey, *P. vivax* and *P. falciparum* infections were found in all  
176 villages across the study region (Figure 1A). The prevalence of *P. vivax* around index infections  
177 decreased threefold from 21.3% at 0 km to the global study prevalence of 6.4% at maximum distance  
178 (Figure 1B). The estimate at 0 km may include household members and inhabitants of the same  
179 building. For *P. falciparum*, prevalence decreased from 12.9% to 3.0%. Within 1 km, it decreased to  
180 13.3% for *P. vivax* infections and to 7.9% for *P. falciparum* infections. Amongst the villages inside the  
181 forest, *P. vivax* prevalence decreased from 38.4% at 0 km to 28.2% within 1 km (Figure 1C). For *P.*  
182 *falciparum*, prevalence fell from 23.9% to 17.3%. For both species, prevalence remains spatially  
183 clustered beyond the null distribution within 1 km amongst the villages inside and outside the forest  
184 but to a lesser degree, if at all, amongst the villages at the forest fringe.



185

186 *Figure 1 Spatial signature of P. vivax or P. falciparum infections in the Cambodian cross-sectional*

187 *survey. Prevalence of infections at increasing distance around index infections. Panel A: Household*

188 *locations in shades of blue, purple, or green per village outside the forest, at the forest-fringe, or*

189 *inside the forest, respectively. P. vivax and P. falciparum infections in red. B: The spatial signature*

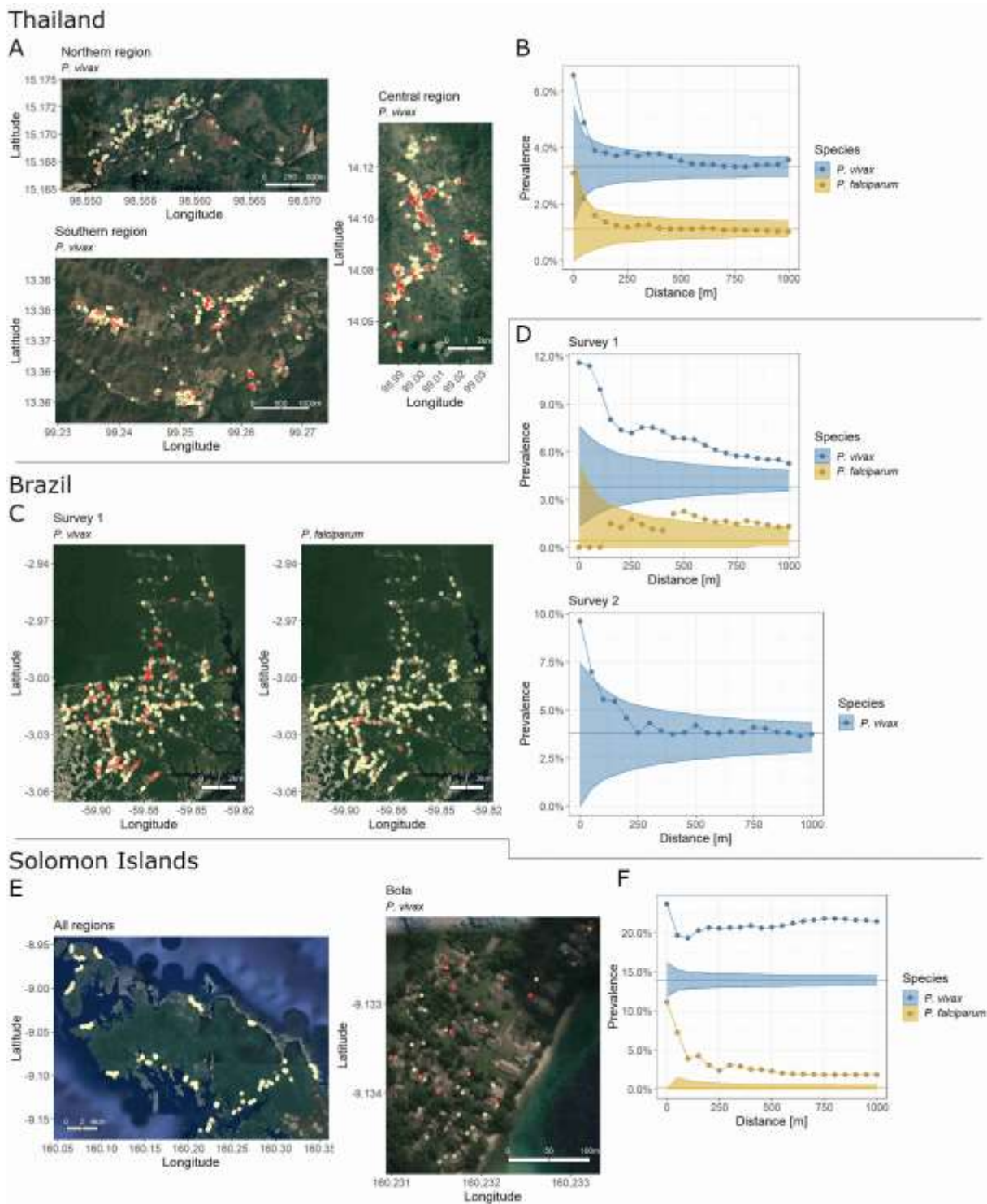
190 *across the entire study region (left) or within 1 km around infections (right). Ribbon: 95%-quantile*

191 *interval of null distribution. Horizontal line: Global study prevalence. C: Stratified by the villages'*

192 *forest proximity within 1 km.*



193 In the cross-sectional survey across three sites in Western Thailand (Figure 2A), prevalence of *P. vivax*  
194 infections around index infections decayed from 6.6% at 0 km to 3.6% at 1 km and for *P. falciparum*  
195 from 3.1% to 1.0% (Figure 2B). Significant spatial clustering for *P. vivax* infections was found only  
196 within a radius of 75 m.



197

198 *Figure 2 Spatial signatures of infections in the cross-sectional surveys in Thailand, Brazil, and*

199 *Solomon Islands. Prevalence of *P. vivax* or *P. falciparum* infections at increasing distance around*

200 *index infections in Thailand (upper panels A-B), Brazil (middle, C-D), and Solomon Islands (lower, E-F).*

201 *Panels A, C, E: Maps with household locations in light gold. *P. vivax* (and for first Brazilian survey also*

202 **P. falciparum*) infections in red. Maps of *P. falciparum* infections in the Thailand survey, maps of the*

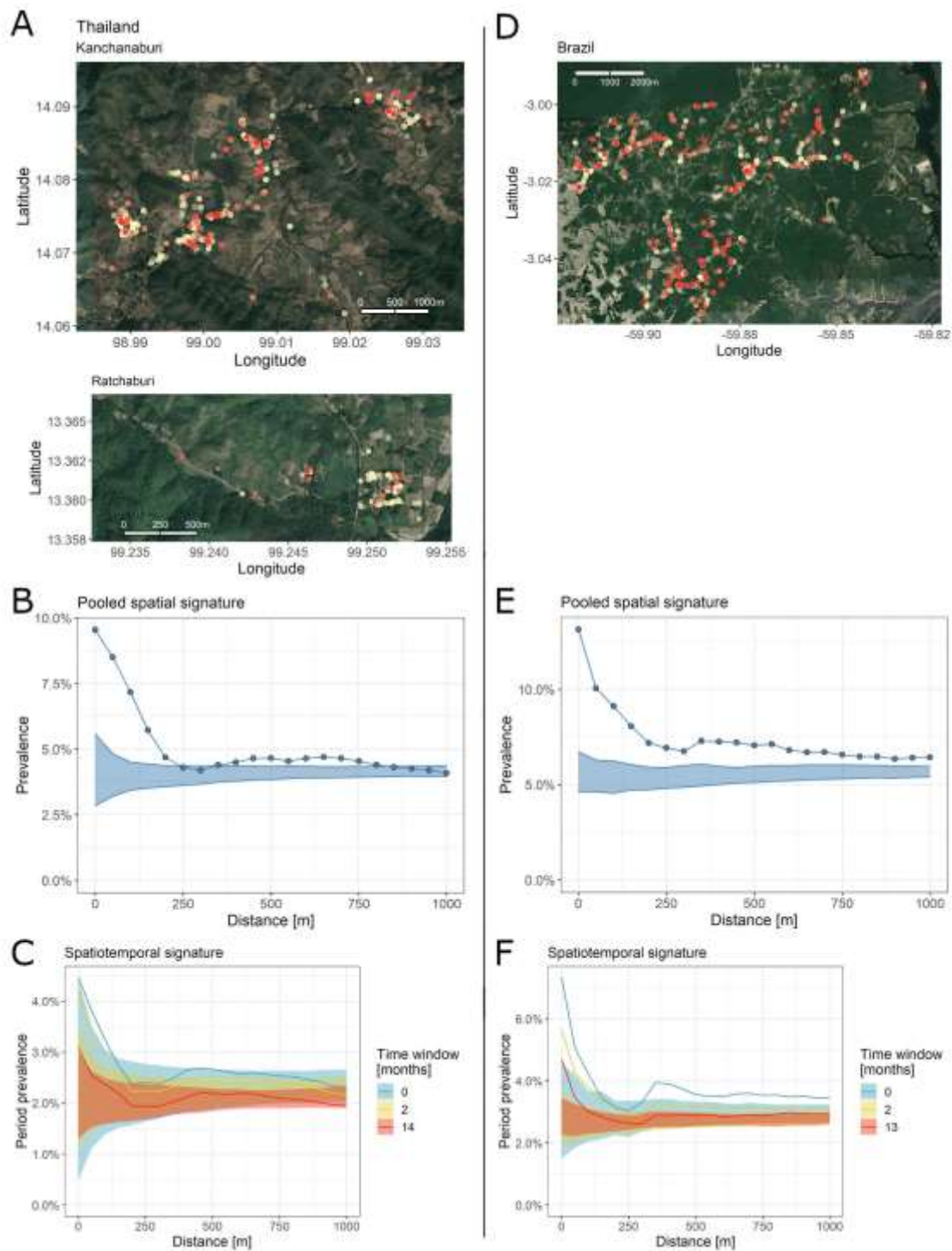
203 *infections in the second Brazilian survey, and complete maps of P. vivax and P. falciparum infections*  
204 *in all surveyed villages in Solomon Islands are in the Supplementary Material. B, D, F: The spatial*  
205 *signatures per survey within 1 km around infections. Ribbon: 95%-quantile interval of null*  
206 *distribution. Horizontal line: Global study prevalence.*

207 In the peri-urban part of Manaus, *P. vivax* and *P. falciparum* infections were detected in the first of  
208 the two cross-sectional surveys (Figure 2C) and *P. vivax* infections in the second survey (maps in the  
209 Supplementary Figure S3). Only one *P. falciparum* infection was found in the second survey,  
210 precluding any signature analysis. *P. vivax* prevalence dropped from 11.4% at 0 km around index  
211 infections to 5.3% within 1 km in the first survey and from 9.6% to 3.7% in the second survey (Figure  
212 2D). Prevalence around *P. vivax* index infections remained higher than the null distribution within 1  
213 km in the first survey and up to 70 m in the second survey. Prevalence of *P. falciparum* infections in  
214 the first survey did not follow a decaying pattern.

215 In the cross-sectional survey in Solomon Islands, *P. vivax* prevalence was 23.7% at 0 km around index  
216 infections and hovered between 19.3% and 21.8% until a distance of 1 km (a decaying pattern is  
217 more apparent from the signature across the entire study region in the Supplementary Figure S5).  
218 For *P. falciparum*, prevalence decreased from 11.1% at 0 km around index infections until 1.8%  
219 within 1 km. Prevalence was elevated from the null distribution for both species across the distance  
220 of 1 km.

## 221 Spatial and spatiotemporal signatures from cohort studies

222 Pooling across the study visits of the longitudinal cohort studies, *P. vivax* prevalence around index  
223 infections per study visit decreased from 9.5% at 0 km to 4.1% at 1 km in the study in Thailand  
224 (Figure 3B) and from 13.1% to 6.4% in the Brazilian study (Figure 3E). The signature remains above  
225 the null distribution until 250 m in the Thai study and entirely within 1 km in the study in Brazil.



226

227 *Figure 3 Spatial and spatiotemporal signatures of infections in the cohort studies in Thailand and*

228 *Brazil. Prevalence of *P. vivax* infections at increasing distance (and in widening time windows) around*

229 *index infections in Thailand (left, panels A-C) and Brazil (right, D-F). Panels A, D: Maps with household*

230 *locations in light gold. *P. vivax* infections in red. B, E: The spatial signatures within 1 km around*

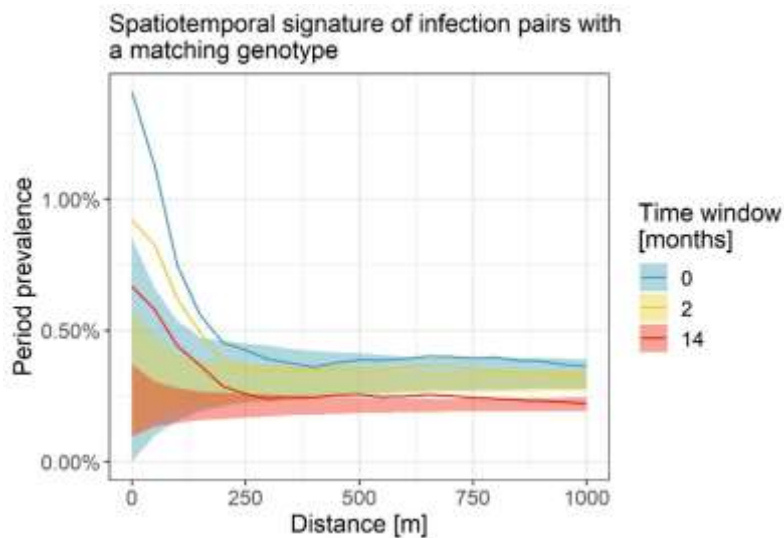
231 *infections, pooling spatial clustering across the study visits. Ribbon: 95%-quantile interval of null*

232 *distribution. C, F: The spatiotemporal signatures of incident P. vivax infections within 1 km and*  
233 *widening time windows in months.*

234 In the cohort study in Thailand, *P. vivax* period prevalence decreased from 4.5% at 0 km around  
235 incident infections to 2.3% within 1 km and the same study month, from 4.3% to 2.3% within 2  
236 months, and from 3.1% to 1.9% across the entire study period of 14 months (Figure 3C). Period  
237 prevalence was above the null distribution until 150 m within the same study month and within 2  
238 months, and 25 m for infections across the study period.

239 In the Brazilian cohort study, *P. vivax* period prevalence fell from 7.3% at 0 km to 3.5% within 1 km  
240 and the same study month, from 5.7% to 3.1% within 2 months, and from 4.7% to 2.9% across the  
241 entire study period of 13 months (Figure 3F). Period prevalence was above the null distribution until  
242 150m within the same study month, until 250 m for infections within 2 months, and 100 m for  
243 infections across the study period.

244 Considering only infection pairs with a matching genotype in the cohort study from Thailand, *P. vivax*  
245 period prevalence decreased from 1.4% at 0 km to 0.4% within 1 km and the same study month,  
246 from 0.9% to 0.3% for infections up to 2 months apart, and from 0.7% to 0.2% across the entire study  
247 period of 14 months (Figure 4). Period prevalence was above the null distribution until 200 m within  
248 the same study month, until 250 m for infections within 2 months, and 250 m for infections across  
249 the study period.



250

251 *Figure 4 Spatiotemporal signatures of infections with a matching genotype in the cohort study in*  
 252 *Thailand. Period prevalence of P. vivax infections at increasing distance (and in widening time*  
 253 *windows) around index infections. Ribbon: 95%-quantile interval of null distribution.*

254 **Stronger spatial clustering at lower global prevalence**

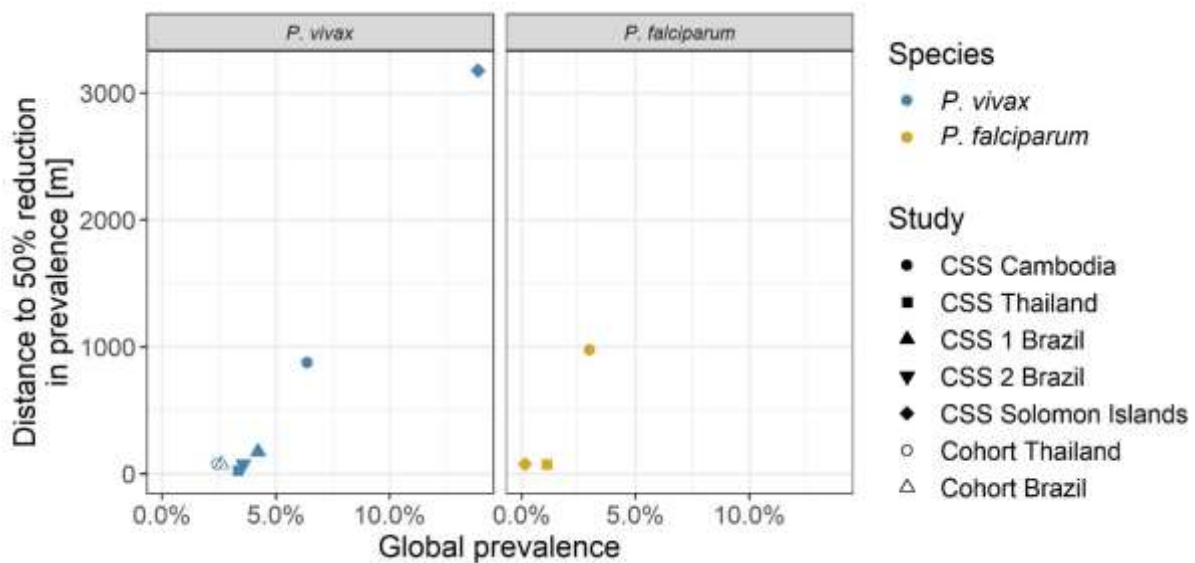
255 Across the spatial signatures of the cross-sectional surveys and cohort studies, the distance within  
 256 which prevalence is reduced by 50% varies from 3,175 m in the Solomon Islands survey to 25 m in  
 257 the Thailand survey for *P. vivax* and from 975 m in the Cambodian survey to 75 m in the Thailand and  
 258 Solomon Islands surveys for *P. falciparum* (Table 2, Figure 5). Overall, the distance to 50% reduction  
 259 tends to be shorter with lower prevalence.

260 *Table 2 Distance to 50% reduction in prevalence and global study prevalence across the studies.*

Study type	Study site	<i>P. falciparum</i>		<i>P. vivax</i>	
		Global study prevalence	Distance to 50% reduction in prevalence	Global study prevalence	Distance to 50% reduction in prevalence
Cross-sectional surveys	Cambodia	3.0%	975 m	6.4%	875 m
	Thailand	1.1%	75 m	3.3%	25 m
	Brazil Survey 1	-	-	4.2%	175 m

	Brazil Survey 2	-	-	3.6%	75 m
	Solomon Islands	0.2%	75 m	13.9%	3,175 m
Cohort studies	Thailand	-	-	2.4%	75 m
	Brazil	-	-	2.6%	75 m

261 *Legend: From the spatial signatures (for cross-sectional surveys) or spatiotemporal signatures at time*  
 262 *window of '0 months', i.e. the same month (for cohort studies) for P. vivax and P. falciparum.*



263

264 *Figure 5 Comparative analysis of distance to 50% reduction in prevalence by global study*

265 *prevalence. The distance within which the prevalence around infections has fallen by 50%, across the*  
 266 *spatial signatures of the cross-sectional surveys and cohort studies. CSS: cross-sectional survey.*

## 267 Discussion

268 Across multiple studies in diverse malaria-endemic regions, we demonstrate clustering of *P. vivax*  
 269 and *P. falciparum* infections in close proximity around index infections, decreasing with distance.

270 Comparing the signature to a null distribution after random re-allocation of infection locations shows  
 271 statistically significant clustering in most settings. In a village in Senegal, we have previously shown

272 spatial clustering of *P. falciparum* infections with this method (14). The finding of

273 spatial/spatiotemporal clustering is in line with previous studies close to the study sites (23, 24, 40,

274 41) or in other malaria-endemic regions (22, 25-28, 30-39, 42-44), deploying the SaTScan scanning  
275 method.

276 We did not find clustering for *P. falciparum* in the first Brazilian cross-sectional survey. Given very  
277 low global *P. falciparum* prevalence in both surveys (6), this could be due to a lack of statistical  
278 power. Considering the distribution of infection locations on the map, almost all infections surround  
279 a forested, sparsely inhabited area in which no samples were collected. The resulting spatial  
280 signature is possibly an artefact due to a peculiar distribution of household locations in an  
281 insufficiently sampled transmission hotspot.

282 The signature of *P. falciparum* infections in the cross-sectional survey in Thailand also lacks statistical  
283 significance, however follows a decaying form. This lack of significance most likely stems from low  
284 prevalence and insufficient statistical power. A significant spatial signature was also not observed in  
285 the Cambodian cross-sectional survey among the villages at the forest fringe. This is most likely  
286 because exposure of inhabitants of villages outside the forest is mainly driven by forest-going  
287 behaviour (9), and vectors in this region are mainly found in the forests (12).

288 *P. vivax* infection distribution in Solomon Islands' cross-sectional survey showed significant clustering  
289 in a flat signature within 1 km distance around index infections, with clustering decreasing at greater  
290 distances. There was substantial variation in malaria prevalence between villages (48). By calculating  
291 a spatial signature pooled across all villages that were sampled in the survey, we potentially miss  
292 significant within-village clustering in high transmission areas.

293 We also show spatial clustering in the cohort studies, particularly within shorter time windows. The  
294 amplitude of the signatures flattens with increasing time windows, demonstrating temporal  
295 clustering. Considering only infection pairs of a matching genotype, the *P. vivax* spatiotemporal  
296 signature in the Thai cohort becomes even more pronounced. While the genotyping methods used  
297 here lack the resolution to define separate transmission chains with certainty, the finding that



298 infections with a matching genotype cluster in space and time corroborates the principle of the  
299 spatial signature method.

300 The study is subject to certain limitations. First, all included studies were based on household  
301 locations. In an area with peri-domestic vector exposure, spatial clustering and a decrease with  
302 distance based on household locations can be readily interpreted, mainly by the vectors' host-  
303 seeking. The interpretation in areas with mainly behavioural exposure is less straightforward, e.g. in  
304 villages outside the forest in the Cambodian site (9). Significant clustering of infections based on  
305 household location could still occur though, e.g. resulting from forest-goers tending to live closer to  
306 each other in the villages or more general clustering of household locations based on socio-economic  
307 factors. The fact that even in these settings clustering might be found is useful from a programmatic  
308 perspective.

309 Another limitation is that the form of the signatures depends on the locations of the households and  
310 villages that were sampled, i.e. the underlying distribution of pairwise distances in the data. Pooling  
311 across villages attempts to generalize the prevalence dynamics. Studies that sampled across adjacent  
312 villages (e.g. the Cambodian survey) are easily accessible for this approach. However, if sampled  
313 villages are far apart, it can complicate the interpretation (e.g. the survey in Solomon Islands). We  
314 controlled for this limitation by ensuring that no gaps occur in the pairwise distance distributions.  
315 However, the *P. falciparum* signature in the first Brazilian survey may be an example where these  
316 distributions cause artefacts in the signature.

317 It is generally acknowledged that malaria elimination efforts need to be spatially tailored towards  
318 areas where (the highest) transmission risk persists (10, 19). The scanning method has proven  
319 effective for detecting and locating clusters of infections. However, the method detects a single or  
320 multiple hotspots, ordered by their likelihood. The localization of hotspots with the strongest  
321 clustering can distract from spatial clustering that also exists outside the statistically most significant  
322 cluster. While the assessment of village-level prevalence and scanning methods are very valuable for

323 identifying local spatial structure (e.g. identifying hotspots), these tools provide limited information  
324 on the more general properties of the spatial nature of malaria infection. Also, while some studies  
325 found 'stable hotspots', i.e. recurring hotspots in the same area from one year to another (36-38),  
326 others did not find that temporal predictive value (32). Repeating resource-intensive observational  
327 studies in order to regularly update hotspot mapping is not feasible for malaria elimination  
328 programs.

329 Reactive interventions are part of the recommended toolbox for malaria elimination (10). Unless  
330 they choose levels such as households or villages, malaria control programmes need to be informed  
331 on the radius around index infections in which to operate. Scanning methods report the spatial  
332 extent of the detected hotspots which is very specific to the investigated area and only partially  
333 generalizable. The spatial signature method allows to detect clustering, to quantify its magnitude,  
334 and shows its dynamics across increasing distance. For that purpose, it generalizes the spatial  
335 clustering across the entire study area and data points. From a programmatic perspective, it can  
336 inform a cost-benefit approach for the optimal selection of a radius around detected index  
337 infections, balancing the trade-off between the total number of households included, and the  
338 expected proportion of infections found. That clustering on the basis of household locations was  
339 found even in areas with occupationally driven exposure is encouraging. From a reactive intervention  
340 perspective, such socio-economic clustering may still allow effective targeting, regardless of the  
341 underlying dynamics.

342 We consider the distance to 50% reduction in prevalence as a suitable measure for comparing the  
343 signatures across study sites. For both *P. vivax* and *P. falciparum*, we observed a trend towards  
344 stronger spatial clustering at lower global study prevalence. This suggests that reactive infection  
345 detection strategies could become increasingly effective in low transmission settings approaching  
346 malaria elimination.

## 347 Conclusion

348 We demonstrate spatial clustering of *P. vivax* and *P. falciparum* infections across a diverse set of  
349 study sites and transmission intensities. Introducing a novel method in spatiotemporal malaria  
350 epidemiology, we quantify the distance within which clustering occurs around index infections. These  
351 distances are often short, e.g. below 200 m, tending to lower values at lower global study  
352 prevalence. The spatial signature of *Plasmodium spp.* infections offers a new tool to extract insights  
353 on *Plasmodium spp.* epidemiology from observational epidemiological field studies. It also provides a  
354 method to inform reactive infection detection strategies regarding effective and feasible radius  
355 choices of interventions around detected infections.

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486

## 487 Figures

488 Figure 1 **Spatial signature of *P. vivax* or *P. falciparum* infections in the Cambodian cross-sectional**  
489 **survey.** Prevalence of infections at increasing distance around index infections. Panel A: Household  
490 locations in shades of blue, purple, or green per village outside the forest, at the forest-fringe, or  
491 inside the forest, respectively. *P. vivax* and *P. falciparum* infections in red. B: The spatial signature  
492 across the entire study region (left) or within 1 km around infections (right). Ribbon: 95%-quantile  
493 interval of null distribution. Horizontal line: Global study prevalence. C: Stratified by the villages'  
494 forest proximity within 1 km.

495 Figure 2 **Spatial signatures of infections in the cross-sectional surveys in Thailand, Brazil, and**  
496 **Solomon Islands.** Prevalence of *P. vivax* or *P. falciparum* infections at increasing distance around  
497 index infections in Thailand (upper panels A-B), Brazil (middle, C-D), and Solomon Islands (lower, E-F).  
498 Panels A, C, E: Maps with household locations in light gold. *P. vivax* (and for first Brazilian survey also  
499 *P. falciparum*) infections in red. Maps of *P. falciparum* infections in the Thailand survey, maps of the  
500 infections in the second Brazilian survey, and complete maps of *P. vivax* and *P. falciparum* infections  
501 in all surveyed villages in Solomon Islands are in the Supplementary Material. B, D, F: The spatial  
502 signatures per survey within 1 km around infections. Ribbon: 95%-quantile interval of null  
503 distribution. Horizontal line: Global study prevalence.

504 Figure 3 **Spatial and spatiotemporal signatures of infections in the cohort studies in Thailand and**  
505 **Brazil.** Prevalence of *P. vivax* infections at increasing distance (and in widening time windows) around  
506 index infections in Thailand (left, panels A-C) and Brazil (right, D-F). Panels A, D: Maps with household  
507 locations in light gold. *P. vivax* infections in red. B, E: The spatial signatures within 1 km around  
508 infections, pooling spatial clustering across the study visits. Ribbon: 95%-quantile interval of null  
509 distribution. C, F: The spatiotemporal signatures of incident *P. vivax* infections within 1 km and  
510 widening time windows in months.

511 Figure 4 **Spatiotemporal signatures of infections with a matching genotype in the cohort study in**  
512 **Thailand.** Period prevalence of *P. vivax* infections at increasing distance (and in widening time  
513 windows) around index infections. Ribbon: 95%-quantile interval of null distribution.

514 Figure 5 **Comparative analysis of distance to 50% reduction in prevalence by global study**  
515 **prevalence.** The distance within which the prevalence around infections has fallen by 50%, across the  
516 spatial signatures of the cross-sectional surveys and cohort studies. CSS: cross-sectional survey.

## 517 [Additional material](#)

518 File name: Additional file 1

519 File format: .docx

520 Title of data: Supplementary figures S1-S5

521 Description of data: Distributions of distance between pairs of study participants or between  
522 pairs of cases and all other study participants across the studies; maps of households and *P.*  
523 *falciparum* infections in the cross-sectional survey in Thailand; maps of households and *P. vivax*  
524 and *P. falciparum* infections in the second cross-sectional survey in Brazil; maps of households  
525 and *P. vivax* and *P. falciparum* infections in the villages of the cross-sectional survey in Solomon  
526 Islands; spatial signature of prevalence of *P. vivax* or *P. falciparum* infections in the cross-  
527 sectional survey in Solomon Islands across the full distance range.

## 528 [List of abbreviatons](#)

CSS	Cross-sectional survey
GPS	Global positioning system
PCR	Polymerase chain reaction

529

530 **Declarations**

531 **Ethics approval and consent to participate**

532 The observational studies from which data were used list their ethics approval references in  
533 the original publications.

534 **Consent for publication**

535 The observational studies from which data were used state the consent for publication in the  
536 original manuscripts.

537 **Availability of data and materials**

538 The observational studies from which data were used list their data availability statements in  
539 the original publications.

540 **Competing interests**

541 The authors declare that they have no competing interests.

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#### 555 *Authors' contributions*

556 MW, IM, and MS conceived and designed the study and methodology and were overall  
557 responsible for the study. WM, ML, WN, JS, AW, AV, BW, LJR, and IM contributed the data.  
558 MS implemented the method and conducted the analyses. MW and IM supervised the  
559 analysis. IM and MW contributed to data interpretation. MS wrote the original draft of the  
560 manuscript. AW, HS, LJR, IM, and MW contributed to the writing. All authors read and  
561 approved the final manuscript.

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**Chapter 5: Fine-scale spatiotemporal mapping of asymptomatic and clinical *Plasmodium falciparum* infections: epidemiological evidence for targeted malaria elimination interventions**

# Fine-scale Spatiotemporal Mapping of Asymptomatic and Clinical *Plasmodium falciparum* Infections: Epidemiological Evidence for Targeted Malaria Elimination Interventions

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**Background.** A detailed understanding of the contribution of the asymptomatic *Plasmodium* reservoir to the occurrence of clinical malaria at individual and community levels is needed to guide effective elimination interventions. This study investigated the relationship between asymptomatic *Plasmodium falciparum* carriage and subsequent clinical malaria episodes in the Dielmo and Ndiop villages in Senegal.

**Methods.** The study used a total of 2792 venous and capillary blood samples obtained from asymptomatic individuals and clinical malaria datasets collected from 2013 to 2016. Mapping, spatial clustering of infections, and risk analysis were performed using georeferenced households.

**Results.** High incidences of clinical malaria episodes were observed to occur predominantly in households of asymptomatic *P falciparum* carriers. A statistically significant association was found between asymptomatic carriage in a household and subsequent episode of clinical malaria occurring in that household for each individual year ( $P$  values were 0.0017,  $6 \times 10^{-5}$ , 0.005, and 0.008 for the years 2013, 2014, 2015, and 2016 respectively) and the combined years ( $P = 8.5 \times 10^{-8}$ ), which was not found at the individual level. In both villages, no significant patterns of spatial clustering of *P falciparum* clinical cases were found, but there was a higher risk of clinical episodes <25 m from asymptomatic individuals in Ndiop attributable to clustering within households.

**Conclusion.** The findings provide strong epidemiological evidence linking the asymptomatic *P falciparum* reservoir to clinical malaria episodes at household scale in Dielmo and Ndiop villagers. This argues for a likely success of a mass testing and treatment intervention to move towards the elimination of malaria in the villages of Dielmo and Ndiop.

**Keywords.** malaria; asymptomatic; clinical malaria; clustering; interventions.

The World Health Organization (WHO) African Region was home to 93% of the 228 million malaria cases and 94% of the ~405 000 deaths attributed to malaria in 2018 [1], despite the dramatic decline of malaria incidence recorded in past decades in many countries [1], which has led in some places to the belief that elimination could be attained.

The move from control to elimination entails the need to shift the primary focus of early detection and rapid case management of clinical malaria cases to the interruption of malaria

transmission including the clearance of asymptomatic infections. In areas where repeated exposure to *Plasmodium falciparum* infections is commonplace, individuals develop nonsterile immunity to clinical malaria [2] that often is associated with a long-term carriage of asymptomatic infections [3–5]. In low transmission areas, asymptomatic *P falciparum* infections have been reported to contribute to 20%–50% of human-to-mosquito infections [6], and parasites from asymptomatic individuals have been reported to be even more infectious to vectors than those from symptomatic cases [7, 8].

In Dielmo and Ndiop, 2 villages situated in the center of Senegal (West Africa) [9, 10], the disease epidemiology has substantially changed to the point that the elimination of the disease is being considered [11–13]. The progress has unfortunately been somewhat hampered by resurgences of malaria attacks [12, 13], and a low-level residual malaria transmission attributed to persistent asymptomatic infections is regularly documented in these areas [11, 14]. Findings from 3 cross-sectional

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surveys performed before the malaria transmission season from 2013 to 2015 identified substantial asymptomatic infections in the communities with average parasite carriage of 7.5% and 9.7% in Dielmo and Ndiop, respectively [11].

The microgeographical analysis of such asymptomatic *Plasmodium* infections in relation with subsequent clinical malaria cases could serve as a rationale to trigger the application of targeted interventions toward the elimination of the disease. In fact, microgeographical analyses can inform how, when, and where clustering occurs and how it relates to transmission patterns [15]. Major elimination strategies such as mass or focused treatment with or without screening and reactive case detection (RCD) rely on being able to identify clusters of infections that can be targeted with appropriate interventions [16].

A recent study conducted at 4 field sites in The Gambia, Mali, and Senegal found separate spatial, temporal, and spatiotemporal clustering of *P falciparum* infections [17]. However, the authors visited participating households only once in The Gambia (rainy season in 2012) and Mali (dry season in 2013), detected asymptomatic *P falciparum* infections by using microscopy only, and did not account for submicroscopic and clinical *P falciparum* infections in the clustering analysis.

The current study capitalizes on cross-sectional-based asymptomatic *P falciparum* infections documented over 4 consecutive years and clinical *P falciparum* episodes of the Dielmo and Ndiop cohorts to explore the relationship between asymptomatic *P falciparum* carriage and subsequent clinical malaria episodes at the individual and household (HH) levels. The study also explored the spatiotemporal clustering and spatial signature of *P falciparum* infections in the communities in both villages.

## MATERIALS AND METHODS

### Study Sites and Samples Collection

The study was conducted in Dielmo and Ndiop, 2 Senegalese villages where malaria has been closely monitored since 1990s [9, 10]. Asymptomatic individuals were recruited before the malaria transmission season through community-based cross-sectional surveys in both villages in July 2013, June 2014, June 2015, and June 2016. No formal sample size calculation was performed because the objective was to sample the largest possible fraction of the project's participants who were present in the villages during the sampling period. A total of 2792 venous or capillary blood samples were provided from asymptomatic individuals during the 4 sampling periods. Each participant was identified with a unique code, and HH in both villages were geo-referenced (Figure S1) using a handheld global positioning system device (GPS 76, Garmin International Inc.). The study falls within the ongoing Dielmo and Ndiop project and was

examined and approved by the Senegalese National Health Research Ethics Committee.

### Detection and Characterization of *Plasmodium* Species by Microscopy and Molecular Methods

The detection of *Plasmodium* spp was carried out using both light microscopy (LM) examination of blood films and quantitative real-time polymerase chain reaction (qPCR) as previously described [18] and detailed in Supplementary Material 1. For the qPCR, DNAs from confirmed *P falciparum*-, *Plasmodium malariae*-, *Plasmodium ovale*-, and *Plasmodium vivax*-infected patients were used as positive controls in all amplifications, whereas sterile water and genomic DNA from uninfected blood samples served as negative controls.

### Survey of Clinical Malaria Episodes

Clinical malaria episodes were surveyed among febrile patients (axillary temperature  $\geq 37.5^{\circ}\text{C}$  or history of fever in the last 24 hours) who presented at the health posts for consultation after the cross-sectional sampling until December of the same year. Patients positive for malaria by rapid diagnostic test and/or LM diagnosis were treated with artemether-lumefantrine as recommended by the National Malaria Control Program. Blood samples were collected from patients for follow-up, and all patients' clinical and epidemiological data were recorded in the Dielmo/Ndiop database at Institut Pasteur, where they were consulted for the purpose of this study.

### Risk Analysis and Spatial Mapping

All data were analyzed using the R statistical software program. Differences in proportions were assessed using Fisher's exact test. Logistic regression was used to estimate the odds ratio for the associations between PCR positivity in a survey and the risk of experiencing an episode of clinical malaria during the rest of the year. Individual-level analyses were adjusted for age, sex, village, and year of cross-section. Household-level analyses were adjusted for household size, village, and year of cross-section.

The previously described concept of spatial signature was used to assess the degree of spatial clustering of *P falciparum* infections and clinical episodes of malaria [19]. The relative risk  $\tau$  of a PCR-positive (or a clinical) case, occurring within a distance  $d$ , of a "neighboring" PCR-positive individual (denoted as the "central" case) in the same year is expressed as  $\tau(d) = \pi(d)/\pi(d_{max})$ .  $\pi()$  is the ratio of the number of pairs of central and neighboring cases within distance  $d$  in the same year as the numerator and the number of pairs of central and neighboring cases within distance  $d$  in the same year as the denominator.  $d_{max}$  is the maximum distance between points in the dataset. Ninety-five percent uncertainty intervals were generated from a null distribution of  $\tau$  after randomly reassigning the clinical and PCR status across the HH locations and time points 1000 times.

## RESULTS

### Characteristics of the Study Population

The 2792 blood samples investigated in this study for the presence of *Plasmodium* parasites were collected in 2013 (N = 611), 2014 (N = 713), 2015 (N = 741), and 2016 (N = 727) (Table 1). The mean and standard deviation of ages of participants were comparable over the 4 periods, and were 21.3 years (19.3) in 2013, 22.1 years (18.6) in 2014, 22.3 years (18.9) in 2015, and 22.3 years (18.8) in 2016 (Table 1). Participants' ages ranged from 0.2 to 93.4 years (Table 1).

### Submicroscopic and Clinical *P falciparum* Infections

The proportions of submicroscopic *Plasmodium* infections and species composition for the 2013, 2014, and 2015 samples were previously reported, and unique *P falciparum* infections accounted for 3.26% (21/611), 11.1% (90/713), and 5.8% (44/741) in 2013, 2014, and 2015, respectively [11]. In 2016, all samples were negative by LM, whereas qPCR detected 11.8% (86/727) positive samples, all of which were due to *P falciparum* (Table 2).

In both villages, the carriage of asymptomatic *P falciparum* parasites was similarly distributed and was age-dependent, with frequencies being higher in older individuals (>15 years) from 2013 to 2015 [11] and in 2016 (data not shown). The number of clinical malaria episodes recorded during the follow-up period was 126 in 2013 (45 in Dielmo and 81 in Ndiop), but decreased progressively over time to 86 in 2014 (49 in Dielmo and 37 in Ndiop), 13 in 2015 (3 in Dielmo and 10 in Ndiop), and 11 in 2016 (3 in Dielmo and 8 in Ndiop) (Table 2). The frequencies of clinical malaria cases were greater in individuals aged 5–15 years (Table 2).

### Mapping of Asymptomatic and Clinical *Plasmodium* Infections

To generate a high-resolution picture of the localization of *P falciparum* infections within HHs in both Dielmo and Ndiop villages, asymptomatic *P falciparum* infections (green spots) and clinical *P falciparum* episodes (red spots) were positioned within their respective HHs (Figure 1).

In Dielmo, the mapping analysis revealed that 85.71% (6/7), 77.3% (17/22), 21.4% (3/14), and 13.3% (2/15) of HHs with asymptomatic *P falciparum* infections during the cross-sectional sampling had clinical malaria episodes in 2013, 2014, 2015, and 2016, respectively (Figure 1). Two and 4 clinical malaria episodes occurred among previous asymptomatic carriers (orange spots) in 2013 and 2014, respectively (Figure 1).

In Ndiop, all HHs with asymptomatic *P falciparum* carriers recorded clinical malaria cases documented on individuals different from asymptomatic carriers in 2013 (Figure 2). The 37 clinical malaria cases of 2014 occurred in 11 HHs, of which only 2 had no previous asymptomatic carriers, whereas 9 occurred in previous asymptomatic carriers (Figure 2). In 2015, 10 clinical malaria cases occurred in 4 households, all of which had previous asymptomatic carriers. In 2016, 8 clinical malaria cases occurred in 6 HHs, 5 of which had previous asymptomatic carriers (Figure 2).

Together, the spatiotemporal mapping of *P falciparum* infections revealed that 68% (160/236) of clinical malaria episodes occurred in HHs where a *P falciparum* asymptomatic case existed.

### Individual Versus Household Risk Analysis in Dielmo and Ndiop

The individual datasets from Dielmo and Ndiop villages collected throughout the 4-year sampling period were pooled and used to investigate the relationship between asymptomatic *P*

**Table 1. Demographic Characteristics of the Study Population**

	2013		2014		2015		2016	
	N = 611		N = 713		N = 741		N = 727	
Total	N	%	N	%	N	%	N	%
<b>Location</b>								
Dielmo	301	49.3	348	48.3	357	48.2	354	48.0
Ndiop	310	50.7	365	51.7	384	51.8	373	52.0
<b>Sex</b>								
Male	249	40.7	327	45.4	340	45.9	311	42.2
Female	362	59.3	386	54.6	401	54.1	416	57.8
<b>Age</b>								
Mean	21.3	...	22.1	...	22.3	...	22.3	...
Range	0.4–91.5	...	0.2–92.5	...	0.2–93.4	...	0.3–82	...
±SD	19.3		18.6		18.9		18.8	
<b>Age groups, y</b>								
≤5	113	18.6	120	16.8	121	16.3	130	17.9
5–15	201	32.9	215	30.2	226	30.5	211	29.0
>15	297	48.5	378	53.0	394	53.2	386	53.1

Abbreviation: SD, standard deviation.

**Table 2. Microscopy and qPCR-Based Parasite Prevalence and Clinical *Plasmodium falciparum* Cases in the Studied Areas and Periods**

No.	2013		2014		2015		2016	
	611		713		741		727	
Villages	Dielmo	Ndiop	Dielmo	Ndiop	Dielmo	Ndiop	Dielmo	Ndiop
N (clinical surveillance)	312	332	353	366	357	385	356	376
N (sample)	301	310	348	365	357	384	354	373
<b>Microscopy</b>								
N	0	0	1	1	1	0	0	0
%	0	0	0.28	0.27	0.28	0	0	0
<b>qPCR (all species)<sup>a</sup></b>								
N	14	9	45	47	25	21	22	64
%	4.7	2.9	12.9	12.9	7.0	5.2	6.2	17.2
<b>qPCR (Pf)</b>								
N	13	8	43	47	24	20	22	64
%	4.3	2.6	12.4	12.9	6.7	5.2	6.2	17.2
<b>Submicroscopic</b>								
N	13	8	42	46	23	20	22	64
%	4.3	2.6	12.1	12.6	6.4	5.2	6.2	17.2
<b>Clinical malaria</b>								
N	45	81	49	37	3	10	3	8
<b>Age groups, γ (%)</b>								
<5	1/54 (1.9)	12/61 (19.7)	5/49 (10.2)	6/72 (8.3)	0/51 (0)	0/70 (0)	0/50 (0)	1/81 (1.2)
5–15	23/112 (20.5)	31/97 (32.0)	19/113 (16.8)	13/104 (12.5)	1/110 (0.9)	5/117 (4.3)	0/106 (0)	4/106 (3.8)
>15	21/146 (14.4)	38/174 (21.8)	25/191 (13.1)	18/190 (9.5)	2/196 (1.0)	5/198 (2.5)	3/200 (1.5)	3/189 (1.6)

Abbreviations: Pf, *Plasmodium falciparum*; qPCR, quantitative polymerase chain reaction.

<sup>a</sup>The qPCR detected no *P. vivax*. *P. ovale* was found as single infections in 2 individuals in 2015, and in co-infections with *P. falciparum* in 1 and 9 individuals in 2013 and 2014, respectively. *P. malariae* was not detected in 2015, but 2 unique cases were detected in 2013 and 2014, respectively.

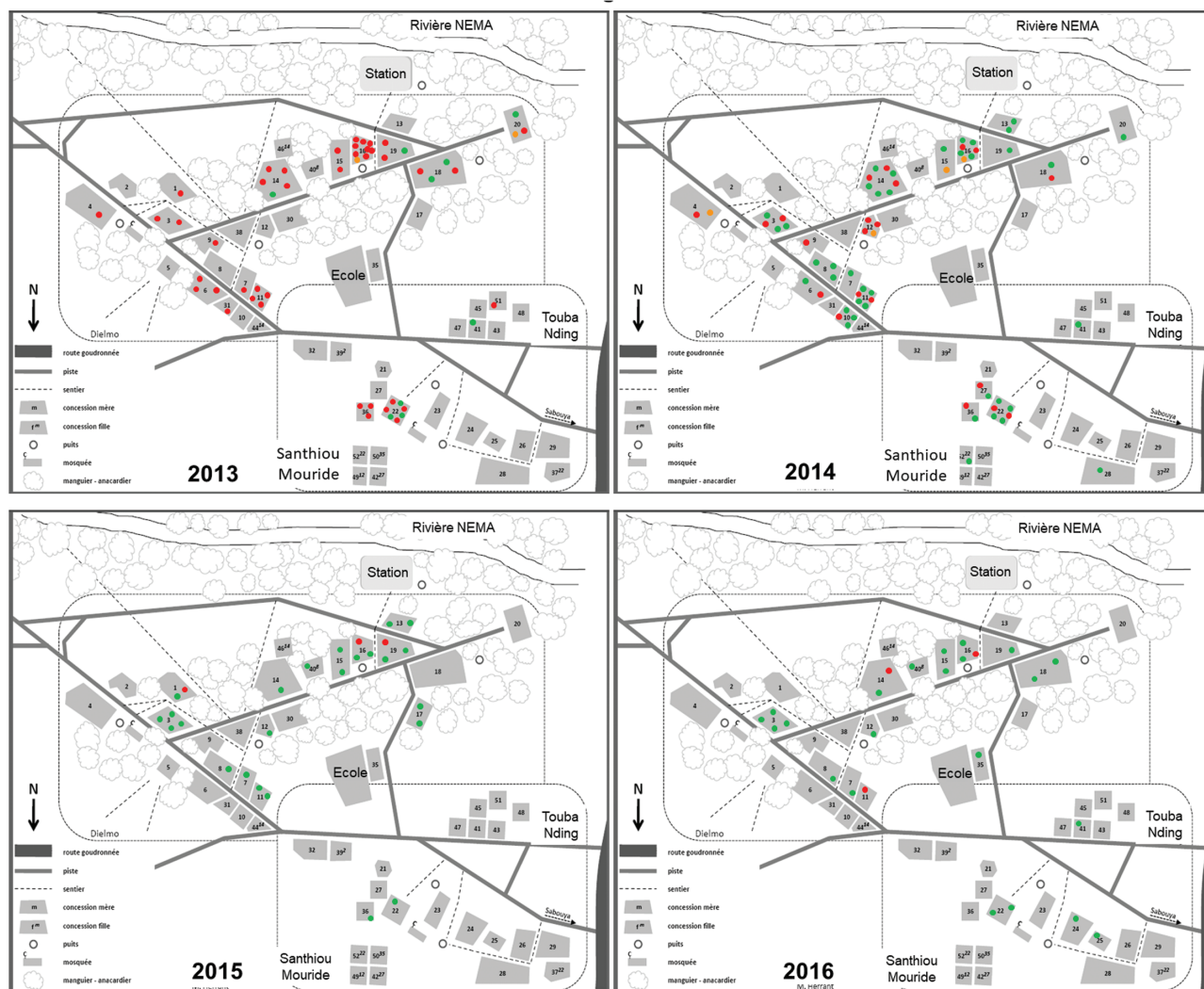
*falciparum* carriage and subsequent clinical malaria episode at the individual and HH levels by applying Pearson's  $\chi^2$  test for statistical difference between proportions. There was no association at the individual level between PCR positivity and the risk of clinical malaria, except for 2014 when PCR-positive individuals had a higher risk of clinical malaria, although this was marginally significant ( $P = .048$ ) (Table 3). At the HH level, a statistically significant association was observed between asymptomatic carriage in a HH and subsequent episode of clinical malaria occurring in that HH (Table 3) for each individual year ( $P$  values were 0.0017,  $6.0 \times 10^{-5}$ , 0.006, and 0.005 for 2013, 2014, 2015, and 2016, respectively) as well as for the combined years ( $P = 8.5 \times 10^{-8}$ ). The individual analysis of the datasets from each setting showed no significant association at the individual level, whereas a statistically significant association was observed at the HH level in Dielmo (Table S1) and Ndiop (Table S2).

The individual and HH risk analysis was adjusted for confounders and the association between *P. falciparum* prevalence by PCR; the subsequent incidence of clinical malaria was reanalyzed using logistic regression (Table 4). If an individual was PCR-positive during a survey, he or she was 1.77 (95% confidence interval [CI]: 1.06–2.93;  $P = .028$ ) times more likely to experience a clinical episode of malaria during the rest of the year. If an individual was PCR-negative during a survey, but lived in the same household as a PCR-positive individual, he or

she was 1.58 (95% CI: 1.12–2.24;  $P = .0097$ ) times more likely to experience a clinical episode of malaria during the rest of the year. If an infection was detected by PCR in a HH, that HH was 2.44 (95% CI: 1.00–5.98;  $P = .0499$ ) times more likely to report a clinical episode of malaria in at least 1 member of the HH (Table 4).

#### Spatial Clustering of *P. falciparum* Infections

The spatial clustering of *P. falciparum* infections can inform the likelihood of infection occurring in nearby people around an individual with asymptomatic or clinical *P. falciparum* infection compared with other people from the same village. The spatial clustering of *P. falciparum* infections was analyzed by determining the proportion of asymptomatic or clinical cases around a PCR-positive individual according to the distance of the infected individual's HH. No significant patterns of spatial clustering of *P. falciparum* infections were found in Dielmo (Figure 3A). This meant that if we identify an individual with an asymptomatic *P. falciparum* infection, nearby people are not more likely to be infected than other people from the same village. By contrast, there was evidence of significant patterns of spatial clustering of *P. falciparum* infections in Ndiop (Figure 3B) (ie, if we identify an individual with *P. falciparum* infection, nearby people are more likely to be infected than other people from the same village).



**Figure 1.** Spatiotemporal localization of asymptomatic (AS, green), clinical (CI, red), and asymptomatic with subsequent clinical (AS + CL, orange) *Plasmodium falciparum* infections within households in Dielmo village from 2013 to 2016.

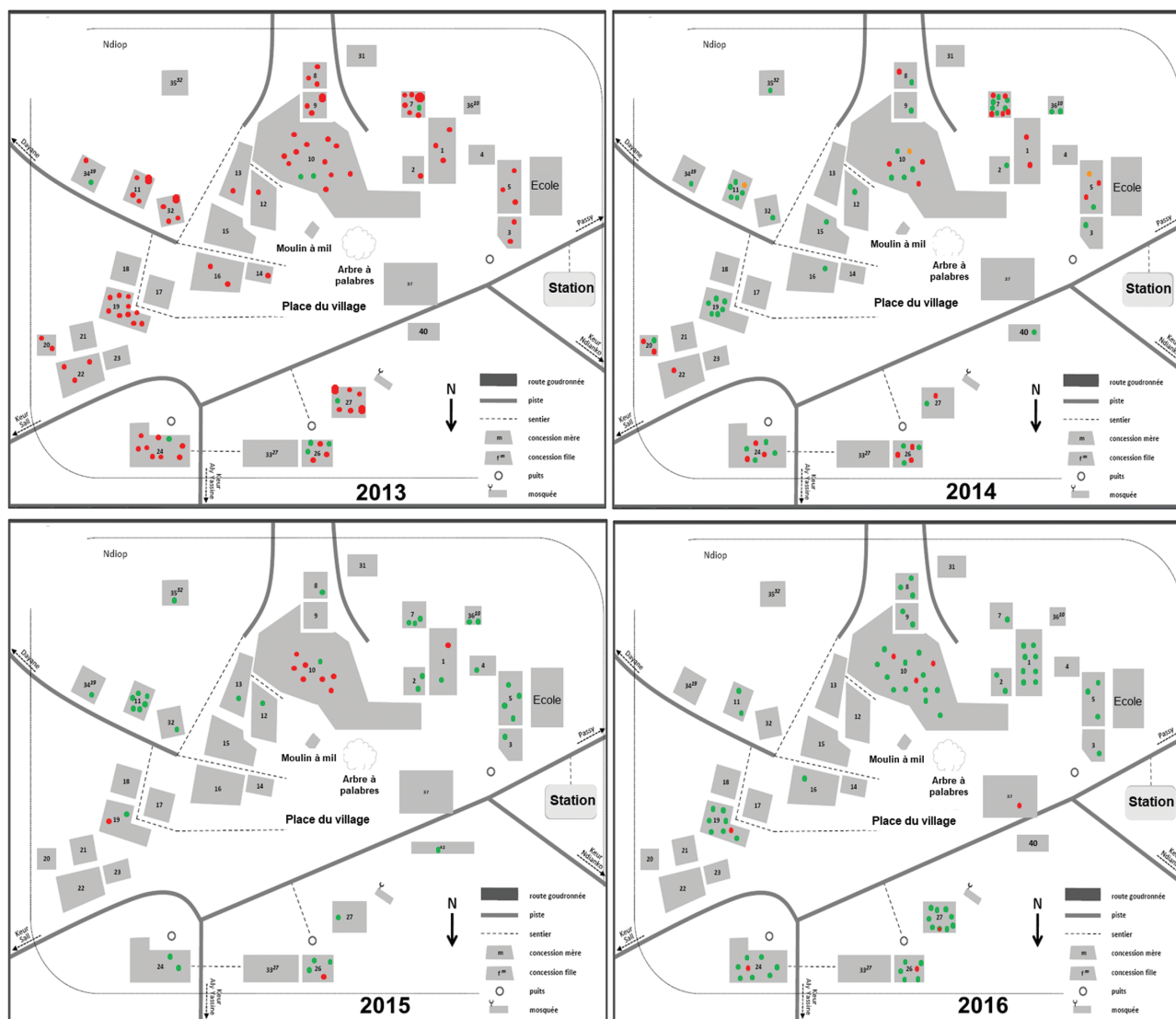
The spatial clustering of *P falciparum* clinical cases revealed no significant patterns of spatial clustering of *P falciparum* clinical cases in Dielmo (Figure 4A) and Ndiop (Figure 4B) (ie, if we identify an individual with asymptomatic *P falciparum* infection, nearby people are not more likely to experience clinical cases than other people from the same village). The higher risk of clinical episodes < 25 m from PCR-positive individuals in Ndiop is attributable to clustering within HHs.

## DISCUSSION

The National Malaria Control Program reported a significant decrease of the incidence of malaria in Senegal [20], allowing the country to embark on elimination programs in parts of the country [21, 22]. The dramatic decline observed in Dielmo and Ndiop has questioned the feasibility of eliminating the disease which would require a reorientation away from the classical control program toward targeted approaches aimed at detecting

and curing all *Plasmodium* infections, as highlighted by WHO [23]. It is largely acknowledged that the elimination strategies in each setting should be based on a detailed understanding of the local epidemiology to guide the application of specific interventions.

In this study, the high prevalence of 11.8% submicroscopic asymptomatic *P falciparum* infections observed in 2016 is in line with the level of residual parasite transmission reported in the previous years in the areas [11]. The high number of clinical *P falciparum* malaria attacks recorded in 2013 was attributed to a rebound following the successful implementation of Artemisinin-based combination therapies in June 2006 and long-lasting insecticidal nets in July 2008 [14], and was associated with the fact that people were exposed to infectious mosquito bites while they stayed late outdoors watching television [24]. Throughout the 4-year survey period, clinical malaria attacks were highest in older children 5–15 years old in both



**Figure 2.** Spatiotemporal localization of asymptomatic (AS, green), clinical (CI, red), and asymptomatic with subsequent clinical (AS + CL, orange) *Plasmodium falciparum* infections within households in Ndiop village from 2013 to 2016.

villages; a pattern attributed to the shift in age susceptibility [12, 28] following long-lasting insecticidal net implementation. Importantly, though the number of asymptomatic and clinical malaria cases varied from 2013 to 2016, the mapping analysis revealed a similar pattern with clinical malaria predominantly occurring in HH of asymptomatic *P falciparum* carriers.

We identified in Dielmo (2013 and 2014) and in Ndiop in 2014 individuals who were asymptomatic carriers of *P falciparum* parasites during the cross-sectional survey and who subsequently experienced a clinical malaria episode during the follow-up period. This could be either the result of newly infecting *P falciparum* clones or the recrudescence of the carried-asymptomatic parasites that have evolved from a sub-clinical to a clinical threshold. The use of parasite genetic data to track infections within and between HHs has been shown

to be critical in the understanding of the extent of within- and between-household transmission dynamics [25–26]. The preliminary genetic relatedness study conducted in Dielmo using *msp-1* genotyping revealed similar genetic profiles between asymptomatic and clinical *P falciparum* parasites from the same individuals and within a HH (Niang et al, unpublished). This suggests that the same parasite populations are circulating within the community in Dielmo, with the asymptomatic reservoir contributing to the occurrence of clinical malaria episodes.

The findings from the risk analysis imply the existence of within-HH transmission with a significant association between the existence of an asymptomatic *P falciparum* infection within a HH and the subsequent occurrence of clinical malaria episodes in HH members of the asymptomatic *P falciparum* carrier, and this was also true at both the Dielmo and Ndiop sites



**Table 3. Individual Versus Household Risk Analysis of the Relationship Between Asymptomatic *Plasmodium falciparum* Carriage and Subsequent Clinical Malaria Episode in Dielmo and Ndiop Villages**

	Analysis at Individual Level					All Years
	2013	2014	2015	2016		
PCR prevalence	34.4% (21/611)	12.6% (90/713)	5.9% (44/741)	11.8% (86/727)		8.6% (241/2793)
Incidence of clinical malaria	19.6% (126/644)	12.0% (86/719)	1.8% (13/742)	1.5% (11/732)		8.3% (236/2837)
PCR status at cross-section	PCR- 15.3% (90/590) PCR+ 14.3% (3/21)	PCR- 10.3% (64/623) PCR+ 17.8% (16/90)	PCR- 1.7% (12/697) PCR+ 0% (0/44)	PCR- 0.8% (5/641) PCR+ 1.2% (1/86)		PCR- 6.7% (171/2551) PCR+ 8.3% (20/241)
P value for difference in proportions	1	.048	1	.53		.35
	Analysis at household level					
PCR prevalence	17.6% (13/74)	55.3% (42/76)	35.0% (28/80)	32.9% (28/85)		35.2% (111/315)
Incidence of clinical malaria	52.7% (39/74)	43.4% (33/76)	8.8% (7/80)	9.4% (9/85)		27.9% (88/315)
PCR status at cross-section	PCR- 44.3% (27/61) PCR+ 92.3% (12/13)	PCR- 17.6% (6/34) PCR+ 64.3% (27/42)	PCR- 1.9% (1/52) PCR+ 21.4% (6/28)	PCR- 3.5% (2/57) PCR+ 25.0% (7/28)		PCR- 17.6% (36/204) PCR+ 46.8% (52/111)
P value for difference in proportions	.0017	$6.0 \times 10^{-5}$	.006	.005		$8.5 \times 10^{-8}$

Fisher's exact test was applied to statistical difference between proportions.  
Abbreviation: PCR, polymerase chain reaction.

when the datasets from each village were analyzed individually or compiled. This is of critical importance because it would suggest that a surveillance system that could detect asymptomatic *Plasmodium* infections during the dry season could also trigger a response to treat all *Plasmodium*-infected individuals in the HH and prevent the occurrence of clinical malaria in the HH.

The efficiency of an RCD strategy for malaria elimination in a given setting likely depends on the optimal screening radius and the number of HHs to investigate following the identification of an index *P falciparum* clinical case. RCD is a detection strategy that takes advantage of the spatial clustering of asymptomatic and/or clinical *P falciparum*-infected individuals by using passively detected cases as triggers to initiate screening and treatment of individuals living in proximity to those cases. The degree of spatial clustering was assessed using the spatial signature of the likely occurrence of asymptomatic and clinical *P falciparum* cases in relation to a previously documented *P falciparum* infection. Beyond the household level, we found significant spatial clustering of *P falciparum* infections in Ndiop but not in Dielmo. Though significant, the degree of spatial clustering in Ndiop was not strong: individuals living with 20 m of another individual with PCR-detectable *P falciparum* infection were 1.35 times more likely to also be infected. Such short-range effects are likely driven by household factors. The effect remained significant at greater distances, with individuals living 50 m of another individual with PCR-detectable *P falciparum* infection being 1.21 times more likely to also be infected (Figure S2). In both villages, there were no significant spatial interactions between PCR positivity in the cross-sectional survey and subsequent risk of episodes of clinical malaria.

An individual's level of immunity may have played a role in maintaining parasites at a subclinical level and contributed to the lack of association found at the individual level. In fact, the asymptomatic carriage has been described as a very complex process associated with low parasite proliferation and an absence of clinical signs related to a specific immunity to soluble antigens and a strain-specific or not antiparasite immunity [27].

Approaches for more efficient malaria elimination interventions have shifted to incorporating spatial dynamics of transmission and to identifying lingering foci. The Framework for Malaria Elimination contained in the WHO Global Technical Strategy for Malaria 2016–2030 included enhanced surveillance, mass drug administration, and active community-based case detection [29]. However, many strategies have encountered variable success in low/moderate transmission settings [30, 31] resulting in either operational challenges of such settings and/or the limited understanding of the heterogeneity in malaria risk and transmission.

## Notes

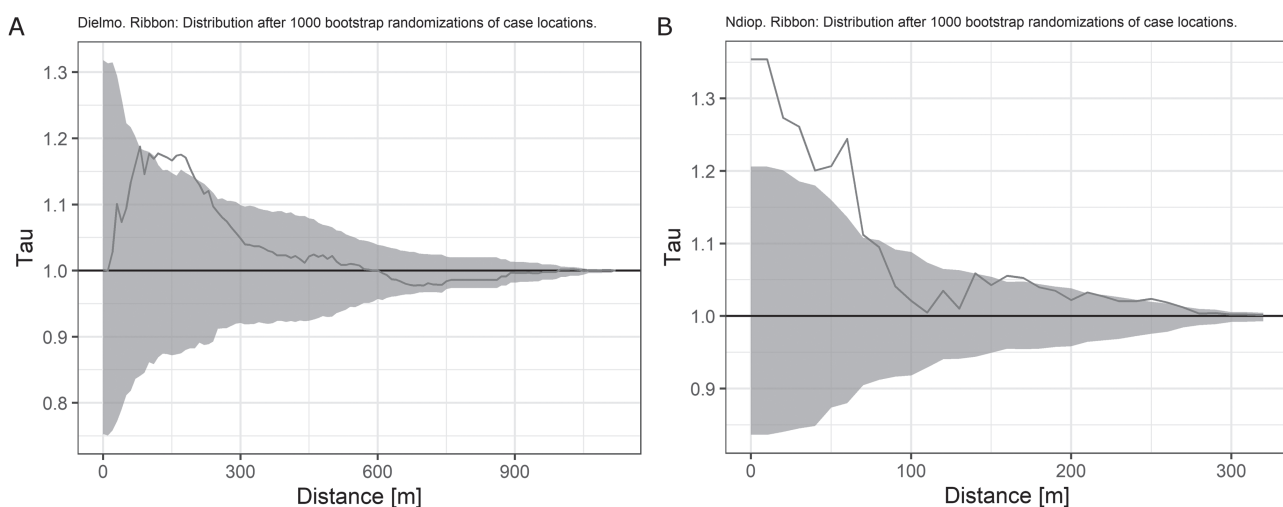
**Author contributions.** M. N. and A. T-B. conceived and designed the study. F. D. S., J. F., A. B., N. D., C. S., and A. T. B. coordinated the fieldwork and samples collection. M. N., A. F. M., R. S., B. D., and L. G. T. performed

**Table 4. Results of Logistic Regression for the Association Between *Plasmodium falciparum* Prevalence by PCR and the Subsequent Incidence of Clinical Malaria**

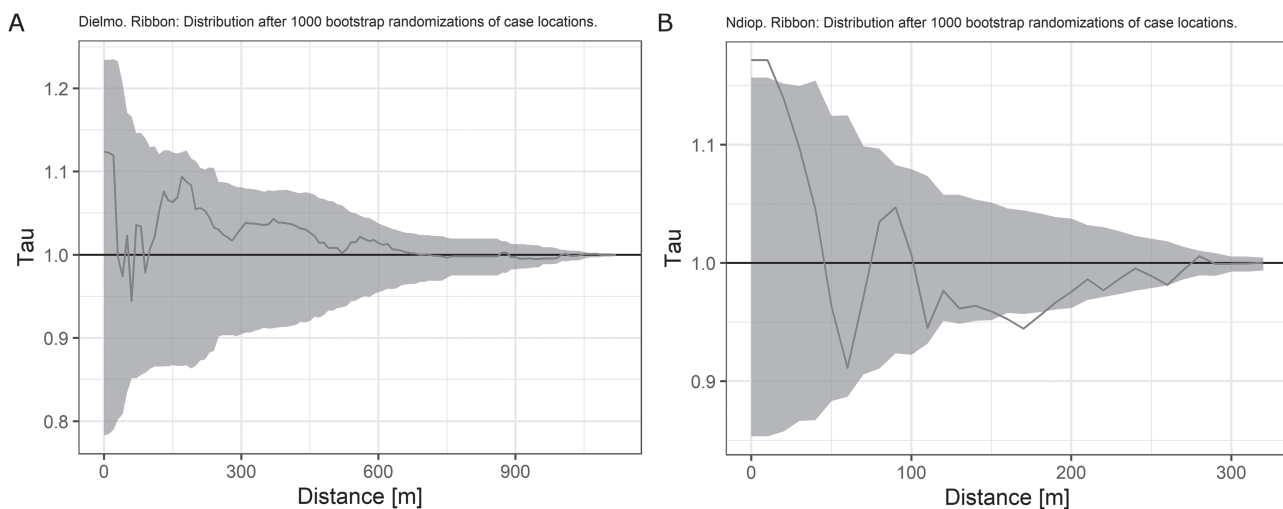
	Pfclin <sub>ind</sub> - PfPCR <sub>ind</sub>		Pfclin <sub>ind</sub> - PfPCR <sub>house</sub>		Pfclin <sub>house</sub> - PfPCR <sub>house</sub>	
	Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted
<b>Dielmo</b>						
OR (95% CI)	1.57 (0.76–3.24)	1.76 (0.83–3.73)	1.95 (1.15–3.30)	2.54 (1.45–4.45)	6.86 (3.33–14.13)	4.51 (1.31–15.62)
P value	.22	.14	.013	.001	$1.7 \times 10^{-7}$	.017
<b>Ndiop</b>						
OR (95% CI)	1.05 (0.54–2.00)	1.78 (0.89–3.55)	0.81 (0.53–1.23)	1.05 (0.70–1.74)	5.05 (2.44–10.45)	1.32 (0.32–5.47)
P value	.88	.10	.32	.66	$1.2 \times 10^{-5}$ *	.70
<b>Dielmo and Ndiop</b>						
OR (95% CI)	1.26 (0.78–2.04)	1.77 (1.06–2.93)	1.19 (0.86–1.65)	1.58 (1.12–2.24)	5.90 (3.54–9.84)	2.44 (1.00–5.98)
P value	.88	.028	.29	.0097	$1 \times 10^{-11}$	.0499

Subscript “ind” denotes the individual level and subscript “house” denotes the household level. Individual-level regressions were adjusted for cross-section, age, sex, and village. Household level regressions were adjusted for cross-section, village, and household size.

Abbreviations: CI, confidence interval; OR, odds ratio; Pfclin, *P. falciparum* clinical malaria; PfPCR, *P. falciparum* PCR positive.



**Figure 3.** Spatial clustering of PCR-positive (asymptomatic) *Plasmodium falciparum* infections in (A) Dielmo and (B) Ndiop villages. Abbreviation: PCR, polymerase chain reaction.



**Figure 4.** Spatial clustering of clinical cases near PCR-positive *Plasmodium falciparum* infections in (A) Dielmo and (B) Ndiop villages. Abbreviation: PCR, polymerase chain reaction.

the laboratory experiments. M. W., M. S., C. T., J. F., and M. N. curated the data and performed the statistical analyses. M. N. and M. W. drafted the manuscript. All authors contributed to the review and editing of the draft manuscript and approved the final manuscript. All authors read and approved the final manuscript and agree to its submission. All relevant data are within the paper. Specific datasets are available from the corresponding author on reasonable request.

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## Chapter 6: Discussion, conclusion, and perspectives

Chapter 6 contextualises the findings from the cross-sectional survey and the spatial signature application within the body of knowledge at the time of the studies and since then. It highlights gaps of knowledge addressed and the expected prospects of the work.

### 6.1. Synthesis

#### 6.1.1. Persisting pockets of high malaria risk on the province and district level

Using PCR with high sensitivity for infections (Canier et al., 2013; Singh et al., 1999; Snounou et al., 1993), numerous cross-sectional surveys have contributed estimates of the prevalence of *Plasmodium spp.* infection in Cambodia (Bosman et al., 2014; Falq et al., 2016; Sluydts et al., 2014; Tripura et al., 2017). Surveys in the western Pailin province in 2013 found infections in 10.5% of participants in the low-transmission season and (with ultra-sensitive PCR) in 15.8% and 20.0% in the high-transmission season (Hustedt et al., 2016; Imwong et al., 2015; Peto et al., 2016). In the dry season in 2016, the prevalence of infection was 8.4% in a survey in the north-eastern Ratanakiri province in 2016, assessed with conventional PCR (Durnez et al., 2018).

Although Mondulkiri, the location of our cross-sectional survey (Sandfort et al., 2020), had surpassed the other provinces in incidence by 2016, most other studies focused on the west (understandably, given antimalarial resistance detected there) or Ratanakiri province in the country's northeast. We estimated a prevalence of *Plasmodium spp.* infection of 8.9% in the low-transmission season and thus found a similar prevalence as Durnez *et al.* in 2016 in Ratanakiri province (Durnez et al., 2018).

The data collected in our survey contributed to further studies by our partners, e.g. a mosquito collection study in the same region that investigated the *Anopheles spp.* behaviour, species composition, the prevalence of insecticide resistance and pathogen carriage (Vantaux et al., 2021). The social, behavioural, and spatial risk patterns also informed a study of GPS-tracked mobility in forest-goers in the same study region (Pepey et al., 2022).

The high prevalence of *Plasmodium spp.* infection in a remote area in Mondulkiri in 2017-2018 was alarming. Together with other surveys, these findings corroborated that malaria persisted in remote provinces in Cambodia. With targets of country-wide elimination in the near distance, these point estimates should aid NMPs with informed milestone evaluations (Cambodian Ministry of Health, 2011; World Health Organization, 2015b).

### **6.1.2. Heterogeneity of infection rates on the village level**

The prevalence of infection is highly heterogeneous below the province level. In the survey in Mondulkiri, we found *P. vivax* and *P. falciparum* infections at a village-level prevalence that ranged between 0.6% and 36.3% for *P. vivax* and between 0% and 25.1% for *P. falciparum*. Variation of prevalence between villages in close proximity has been found in other cross-sectional surveys; however, not at an equally wide range. In the western Battambang province in the dry season in 2015, a survey of 20 villages found no infections in some villages (sampling only 50 people per village). If infections were found, the prevalence of *P. falciparum* infection varied between 2% and 6% and *P. vivax* prevalence varied between 2% and 26%. Infection prevalence was found at lower extremes, although an ultra-sensitive PCR was used in the study (Tripura et al., 2017). Falq *et al.* detected infections in the northern Preah Vihear province in 2014 at a prevalence of 0-4.6% for *P. falciparum*, 0-3.6% for *P. vivax*, or 0-12.1% for *Plasmodium spp.* across all 21 villages of one district, despite the use of ultra-sensitive PCR and sampling in the rainy season (Falq et al., 2016). Only a survey in Ratanakiri in the rainy season more than 15 years earlier found a more extreme village-level infection prevalence, ranging less widely though (Steenkeste et al., 2010). Between-village variation in prevalence was also described in Thailand (Sattabongkot et al., 2018) and Laos (Iwagami et al., 2017; Phommasone et al., 2016; Pongvongsa et al., 2016).

Our survey in Mondulkiri added a unique observation to the literature on infection prevalence patterns in Cambodia as it sampled from all villages in a study area, with relatively high sample sizes, and with villages along a gradient of proximity to the forest. We categorized them along this gradient based on forest cover as deduced from remote sensing analysis of satellite imagery, an objective approach no other surveys in this area have used. The trend of increasing village-level prevalence with closer proximity to the forest was evident and statistically significant. In the survey by Tripura *et al.* from 2015 in Battambang province, a

similar trend of village-level prevalence and proximity to the forest is apparent from the map. However, it is not analytically tested (Tripura et al., 2017). Incardona *et al.* sampled from randomly selected villages in three provinces in 2001-2003 and provided maps of the villages, their infection prevalence based on LM, and forest-covered areas (drawn from aerial and satellite photographs and field verifications). Prevalence tended to be high in remote, forested, mountainous areas or camps outside villages at the forest fringe (Incardona et al., 2007).

The subsequent analysis of the survey in Battambang focused on behavioural exposure by spending time in the forest without accounting for the baseline exposure when residing close to the forest (Tripura et al., 2017). Incardona *et al.* accounted for distance to the forest in their multivariable regression model for infection risk and found an association in two of the three provinces (Incardona et al., 2007). However, they could not simultaneously account for behavioural exposure. Our survey demonstrated by multivariable mixed-effects logistic regression that both behavioural exposure (i.e. working in or travelling to the forest) and living in villages at the forest fringe or inside the forest were independent risk factors of infection.

Since the publication of our survey, no further surveys assessing infection risk in the general population have been published for Cambodia. Our approach offers a methodological example for future studies so that the contribution of residential baseline risk vs. behavioural exposure to infection risk in these persistent pockets of malaria transmission in overall elimination settings can be further distinguished.

### **6.1.3. Spatial clustering of infections beyond the resolution of villages**

In a survey of 117 villages over an area of approximately 80 km in Ratanakiri in 2012, Sluydts *et al.* found hotspots of infection risk with *Plasmodium spp.* in general, and by species (Sluydts et al., 2014). These hotspots were located mainly at a river, but locations varied per species. The hotspots had a 24-32 km radius and were based on the villages' coordinates, i.e. could not stratify infection risk beyond the village scale by design. By contrast, in three villages in Ratanakiri in the dry season in 2016, Durnez *et al.* searched for spatial clusters of PCR-detectable infections per village separately, based on GPS coordinates per household (Durnez et al., 2018). Clusters of *P. vivax*, *P. falciparum*, or *P. malariae* infections could be located

among field houses in densely forested areas and inside two villages close to the forest. These hotspots were between less than 100 m to 1.8 km wide. One cluster in a village included only one house inhabited by numerous families, which could hint at social clustering in the village. Spatial clustering of infections on sub-village-level elsewhere in the GMS was shown by Parker *et al.* in a village at the Thailand-Myanmar border in 2011-2012, where cases clustered in households close to a water drainage system and other continuous water sources (Parker *et al.*, 2015). This survey was, interestingly, the only one from other GMS countries that used the SaTScan method to assess spatial clustering based on household locations.

In our study, household-level GPS coordinates also allowed for the detection of clustering below the village level and yielded two clusters of *Plasmodium spp.* infection. These included households from all study villages located inside the forest. In contrast to the surveys discussed above, detecting artificial spatial clusters due to social clustering was avoided as the analysis was adjusted for non-spatial, behavioural risk factors. That the hotspots remained significant despite the adjustment could indicate additional peri-domestic risk of infection in these villages.

Further studies are needed to underpin the hypothesis of peri-domestic infections in these areas, e.g. assessments of mosquito biting activity throughout the day, similar to the study by Vantaux *et al.* (Vantaux *et al.*, 2021). Future studies are also needed to quantify the differential contribution of behavioural vs. peri-domestic exposure in more detail. Under the assumption of peri-domestic exposure, it remains unclear whether in-village host-vector contact patterns are dense enough to sustain peri-domestic transmission. Alternatively, the transmission system could depend on parasite ‘importation’ by individuals infected due to behavioural exposure, e.g. forest goers. More comprehensive infection control in these risk groups would then also drain the transmission system in the villages. This would probably require genotyping or sequencing studies, which are resource-intensive but have suggested near-clonal circulating parasite lineages elsewhere (Salla *et al.*, 2020).

#### **6.1.4. The use(fulness) of spatial clusters to guide malaria elimination**

The increase in country-wide malaria incidence in Cambodia 2016-2018, see section 2.1, was responded to not only by reinstating the VMW programme and changing the first-

line ACT. The NMP also launched the *Intensification Plan* that ran from 2018 until 2020 and targeted areas and populations with increased malaria risk with further scaled-up testing and treatment by VMWs/MMWs. The clustering of infections at the village or the sub-village level and in hard-to-reach populations, as described in our and other cross-sectional surveys, explicitly informed the programme (Sovannaroeth et al., 2022; Stratil et al., 2021).

The concept of transmission ‘hotspots’ is based on the idea that in every endemic setting, small clusters of households exist that have an increased risk of transmission of *Plasmodium spp.* infection due to local circumstances that facilitate transmission, e.g. proximity to breeding sites (Bousema et al., 2012). From these overly active transmission systems, infections ‘spill’ outwards into the surroundings and maintain transmission there, particularly at overall low transmission levels. Likewise, transmission cycles can persist over the low transmission season within these hotspots and cause expanding transmission with the onset of the high transmission season. Targeting such hotspots would be efficient in reducing infection levels. Malaria control and elimination programmes would thus need to survey intervention areas once, detect the hotspots, and target their interventions to them.

This concept presumes the stability of such hotspots over time. Some studies indeed found areas that were repeatedly hotspots in consecutive surveys. In two surveys one year apart among four villages in Tanzania in the dry seasons of 2010 and 2011, being in a *P. falciparum* hotspot in 2010 was predictive for PCR-detected infection in the second survey (Mosha et al., 2014). In a study from Sudan, consecutive surveys from 1999 until 2008 among the general population in one state found significant hotspots in six of the ten yearly surveys (Nourein et al., 2011). In another study from Sudan, children from 88 villages in a low transmission area were tested for *P. falciparum* infection by LM in one survey per year each January 1999-2009. Hotspots were detected in all eleven yearly surveys, and the hotspots from ten years overlapped with a spatiotemporal cluster based on the entire study period (Mirghani et al., 2010). However, the hotspots drastically varied in size, from containing only a single village to a diameter of more than 60 km. Also, the ten hotspots that overlapped with the space-time cluster did not all overlap, and the variation (regarding the hotspot size and location) was erratic. In a two-year cohort study among children from villages surrounding a hydropower dam in Ethiopia in 2008-2010, the hotspot for *P. falciparum* malaria clinical case



incidence was stable over the two study years. A hotspot of *P. vivax* malaria cases was found in each study year but on opposite sides of the study area (Seyoum et al., 2017).

Studies from the GMS continue to cast doubt on the stability of (local) hotspots. The survey in Ratanakiri province, Cambodia, in the dry season in 2012 detected a hotspot of PCR-detected *P. falciparum* infection that was also found in another survey in the rainy season of the same year. However, the area was not a hotspot of PCR-detectable infections in a consecutive survey one year later. The same holds for a hotspot of *P. vivax* infection detected in the dry and rainy seasons in 2012. A *P. vivax* hotspot detected in 2013 was at a different site. Hotspots based on clinical case incidence were in yet different locations for *P. falciparum* and would not have predicted the hotspot of *P. vivax* infections in 2013 (Kerkhof et al., 2016; Sluydts et al., 2014). In two consecutive surveys among households from 3 villages in Ratanakiri province in 2016 and 2017, living in a household in a hotspot of PCR-detected infection in 2016 was not associated with an individual or household-level risk of infection at the follow-up survey one year later (Bannister-Tyrrell et al., 2019). Furthermore, a cluster-randomized trial of hotspot-targeted interventions in Kenya could not observe evidence for the effect that reducing malaria transmission in the hotspot would also reduce transmission in the surrounding area (Bousema et al., 2016).

Applying the scanning method across a wide area based on village coordinates can lead to hotspot detections that are limited in their usefulness for malaria control and elimination. For example, in the Ratanakiri survey in 2012, one hotspot was a single village with high village-level prevalence, with no extra benefit to conventional village stratification. Other hotspots were vast in geographical extension, some of which contained relatively few infections, e.g. the *P. ovale* and *P. malariae* clusters were 32 km and 30 km wide in radius and contained 13 cases and 18 cases, respectively (Sluydts et al., 2014).

Even assuming that detected hotspots would have predictive value of future infection and thus define suitable targets for control interventions, it would be resource-intensive for an NMP to survey the entire operational area at sufficient coverage for detailed hotspot mapping, potentially even multiple times over the years. The spatial scanning method also does not generalize hotspot formation. It is unlikely that it would be possible to find common denominators of a set of hotspots in order to extrapolate hotspots in other areas without the

need to derive them from detected infections, given the complex interplay of host, mosquito, and pathogen characteristics that make up favourable niches of clustered transmission. Thus, it remains to be demonstrated that spatial scanning for infection hotspots can cost-effectively direct malaria control and elimination interventions and reduce transmission.

Until then, interventions targeting areas of high clinical incidence in the surveillance data and areas populated by known risk groups for infection remain the most practicable guidance for geographical resource allocation by NMPs. However, this stratification of interventions should also be informed by data on the reservoir of sub-patent or asymptomatic infections, as areas of high transmission can have exceptionally high rates of these low parasitaemia infections due to the built-up of immunity. This data would also require (repeated) surveys with active case detection based on molecular detection of infection across the operational area, though at less coverage than hotspot mapping would depend on.

#### **6.1.5. Generalizing spatial clustering of infection risk by the spatial signature method**

SaTScan has proven powerful to detect areas of increased risk of observed outcomes, i.e. to localize (albeit potentially transient) hotspots of parasite carriage, and thus to demonstrate heterogeneity in infection risk on small spatial scales. However, it does not enable to generalize such spatial clustering and thus transfer it to other locations. Also, the radius of areas with increased risk varies greatly depending on the setting and underlying location patterns and thus cannot be generalized. Furthermore, focusing on a detected hotspot bears the risk of losing sight of ongoing transmission chains outside the hotspot within active foci. The method can detect multiple areas with statistically significantly increased risk, but only the highest likelihood cluster is usually reported. Also, the hypothesized effect of reductions outside hotspots, if the transmission is hit inside them, has not been underpinned by statistically significant evidence.

We have tried to overcome these methodological blanks by trying to answer the question of how far, on average, around infections (index infections) one would need to search for co-clustering infections (neighbouring infections). For that purpose, we introduced the spatial signature method to malaria epidemiology. It was originally developed and applied

to dengue fever, HIV/AIDS, and measles (Lessler et al., 2016; Salje et al., 2012). Its initial development was designed to infer spatial clustering from case locations without knowing the spatial distribution of the underlying population from which the cases arose. Instead, it used data on the genetic relatedness of the virus strains of the cases. Spatial clustering was inferred from cases closer to each other having a higher chance of being infected with more closely related pathogen strains.

In malaria surveys, the genetic relatedness of the parasite strains of infections by *P. falciparum*, *P. vivax*, or other species is laborious to determine and rarely reported. However, an underlying population in which the cases were detected is usually known. Hence, we can assess the pooled prevalence of neighbouring infections at a certain distance from index infections and how it develops with increasing distance, see Chapter 4. By randomly re-allocating case locations, statistical significance can be assessed by comparing the observed signature with a null distribution. In that way, several purposes are fulfilled: 1) Spatial clustering is tested for and detected if case pair prevalence is elevated at a close distance above the null distribution, 2) the magnitude of the spatial clustering is quantified by the increase of prevalence with a shorter distance, and 3) the clustering is assessed on the continuous scale of distance between observation pairs. Spatiotemporal patterns can be assessed by comparing pairs of observations sampled at different times. As demonstrated, spatial and spatiotemporal clustering of PCR-detectable infection risk existed to differing degrees across a diverse set of transmission settings from Cambodia, Brazil, Thailand, and Solomon Islands. At medium to low prevalence, the distance to a 50% reduction in prevalence was within 1 km, often even below 100 m around index cases, with a trend to lower distances at lower global study prevalence. That is, infections tend to cluster more in lower transmission settings. Overall, similar distance ranges within which most of the risk reduction occurs were observed in the studies of dengue fever, HIV/AIDS, and measles (Lessler et al., 2016; Salje et al., 2012).

In our study in the two Senegalese villages of Ndiop and Dielmo, we had the chance to apply the spatial signature method in a Sub-Saharan setting and assess the degree of spatial clustering of PCR-detectable *P. falciparum* infections and clinical malaria episodes (Niang et al., 2021), see Chapter 5. Based on blood samples from the general village populations from

each year from 2013 until 2016 and household GPS locations, PCR-detectable infections were found to spatially cluster in Ndiop within 70 m around index infections but not in Dielmo. Clinical cases were not found to spatially cluster beyond the household level.

When applied to the monthly time points of cohort studies of PCR-detectable *P. vivax* infections in Thailand and Brazil, the dilution of spatial clustering of pairs of incident infections (first detections of an infection in a given individual) with widening temporal windows indicated temporal clustering. However, in both settings and particularly in the Brazilian study, the elevated period prevalence of incident case pairs at close proximity was still statistically significant when assessed across the entire year-long study period, indicating the predictive potential of detecting nearby cases around the location of index cases across a year in time.

#### **6.1.6. Spatially informing (re-)active case detection schemes**

As the major recent intervention scheme to accelerate progress towards the malaria elimination targets, the Cambodian NMP launched the *Intensification Plan* (Sovannaroeth et al., 2022). On top of the already established malaria control and intervention activities, the plan contains a further scale-up of VMW and MMW coverage and activities. In identified hotspots, VMWs ought to fill gaps in households owning LLINs and their hammock versions, continue to provide RDT testing and effective ACT treatment, and ensure immediate referral of severe malaria cases to the nearest health clinic, all among the general village populations. Among mobile and migrant populations, MMWs should also provide RDT testing and effective treatment but also actively search for cases that otherwise might not have reported themselves to the MMWs. Firstly, the plan includes active test and treat outreach twice per month to individuals in forest hotspots. Secondly, MMWs are supposed to ask positive forest goers for their co-travellers to test them for infection and treat them if necessary. No active case detection is intended for the village-level, VMW-guided layer of the intervention.

The more homogeneously distributed infection risk across the entire village population in forest villages in our Mondulkiri cross-sectional survey, as well as the short-range distance clustering of infections in our spatial signature analyses, suggest that such an active case detection approach could be helpful in tackling within-village transmission cycles. It would be helpful to repeat the spatial signature analysis on survey data from various regions with high

levels of persistent transmission in Cambodia to assess the generalizability of specific estimates of distances within which infections cluster to a certain degree. Based on this, the NMP would then be able to make an informed choice at what distance from index cases to search for other cases, balancing the expected share of clustering infections that would be covered against the feasibility, i.e. the number of households that would need to be screened. As Kunkel *et al.* argue that the sensitivity of the testing by RDTs is insufficient (Kunkel et al., 2021) and PCR detection is not feasible at the VMW points of care, a spatially informed drug administration scheme could be considered: Families who reside within a certain proximity around index cases are offered treatment without certainty of actual current infection, in analogy to the intermittent preventive treatment of forest goers suggested by Kunkel *et al.* and Dysoley *et al.* (Lek et al., 2020).

Once transmission has been largely interrupted in an area, e.g. by the *Intensification Plan* interventions, malaria elimination programmes need to switch to responsive approaches where few infections are rapidly detected, reported, treated, and followed up. In China, for example, the success in the late stages towards malaria elimination was also attributed to their 1-3-7 surveillance, i.e. reporting confirmed cases within one day, investigating cases detected by RDTs within three days, and applying targeted control responses within a week. In Cambodia, an adapted approach was used in an operational district near-elimination, including active case detection of co-travellers, household and family members, as well as surrounding households (Kheang et al., 2020). Spatial distances provided by evidence derived from spatial signature analyses could also contribute to the parameters for this intervention and thus allow further accelerating towards malaria elimination in a feasible and cost-effective manner.

## 6.2. Conclusions

The enormous effort of routine surveillance of clinical cases from health care facilities and VMWs/MMWs allows for monitoring malaria incidence and comparing countries or regions within a country. This data confirms the remarkable reductions in malaria transmission over the last two decades in Cambodia and the GMS overall. As country-wide incidence has been reduced, the landscape of persisting transmission has become increasingly fragmented, and the remaining burden is found in more and more remote areas. Field studies in such areas, like our survey, further reveal the extent of high local transmission levels. These findings support the increase of peripheral access to malaria diagnosis and treatment via the establishment and recent scale-up of the VMW programme by the Cambodian NMP.

Beyond geographical fragmentation of malaria incidence, infection risk is highly heterogeneous on the individual level. As corroborated by our survey, travelling to or working in the forest is the primary behavioural risk factor for infection. Thus, the MMW initiative of the Cambodian NMP and its focus on forest goers appears well targeted. The predominance of *P. vivax* infections affirms the roll-out of radical cure in combination with G6PD testing.

Clinical incidence relies on the self-reporting of patients to health care facilities, VMWs, or MMWs in reach, proper diagnosis, and onward notification. The vast majority of asymptomatic infections, as found in field studies with active outreach and molecular parasite detection, means that most infections are missed by curative health care. Even pro-active testing by VMWs or MMWs has a limited effect when diagnosis by LM or RDTs is not sensitive enough to detect the sub-patent, low-parasitaemia infections, which nevertheless contribute to transmission.

Our survey confirmed the disproportionate association of infection risk with time spent in the forest. However, residents of villages in the forest were at high risk across all ages and gender, and households from these villages formed hotspots of infections despite adjustment for forest-going behaviour. This suggests peri-domestic transmission and that targeting high-risk groups and passive case detection might not suffice for elimination. As the high prevalence calls for burden reduction and risk is high across the general population, the NMP may need to consider MDA or mass testing and treatment.

Spatial scanning methods like SaTScan are powerful for scanning study areas for statistically significant clusters of positive observations, e.g. hotspots of PCR-detected cases. Their use in spatial malaria epidemiology has been embedded in the hypothesis that certain locations are particularly favourable for transmission and spill infections into neighbouring areas that otherwise would not sustain transmission. Breaking transmission in these hotspots would also halt the spread beyond the intervention area. However, studies have found hotspots not stable over time or not predictive for future infection. A trial did not yield evidence for incidence reduction outside the intervention area. Furthermore, hotspot detection locates clusters but does not generalize spatial dynamics in a given area. Conducting resource-intensive surveys in all areas of operation does not seem feasible for NMPs.

The spatial signature method calculates the pooled prevalence of infections in widening areas (and potentially time windows) around other infections. The method detects clustering with statistical significance, summarizes the extent of clustering, generalizes spatial dynamics in infection risk across the observed area, and evaluates the radius around infections within which other infections occur at elevated likelihood. It revealed spatial clustering of household locations of PCR-positive individuals within close proximity across several field studies, notably in Brazil, Thailand, and Cambodia. The clustering tended to occur in narrower areas around infections at decreasing overall prevalence in the area. Infections in cohort studies appeared to cluster spatially and in time over a few months. These findings could support reactive interventions, such as reactive drug administration or reactive testing and treatment, and enable NMPs to make informed choices of operational distances.

The countries of the GMS have committed to eliminating malaria until 2030 or earlier. Cambodia aims at the overall elimination of malaria by 2025 and had planned to eliminate *P. falciparum* by 2020. The challenges that elimination programmes in Cambodia, the GMS in general, but also other regions face are primarily: the high prevalence of asymptomatic, sub-patent infections, the difficulties of curing *P. vivax*, and the spatial fragmentation of infection risk. These roadblocks to malaria elimination are well established but must be further delineated. As milestones are missed (such as *P. falciparum* malaria elimination in Cambodia by 2020) and threats such as drug resistance are looming, the need to better inform malaria elimination programmes and render them more efficient with novel tools is apparent.

### 6.3. Perspectives

A better understanding of how relapses contribute to *P. vivax* transmission remains vital. Membrane feeding studies suggest infectivity of asymptomatic infections in field studies, and mathematical models reconstruct such dynamics (White et al., 2014; White et al., 2018). Tools to disentangle relapses from new blood-stage infections in longitudinal studies and analyses of their transmission share would be illuminating. Generally, further cohort studies need to shed light on transmission dynamics. In the Asia-Pacific ICEMR, such a study took place in the same region as our survey and will assess spatiotemporal infection patterns.

Data on infection and household location will allow to fit mathematical models of *P. falciparum* and *P. vivax* transmission and extend them to be spatially explicit. These can then compare approaches to identify and hit pockets of residual transmission, e.g. active vs. passive designs – so that elimination is reached most effectively.

Disillusion grows that elimination can be achieved with the insufficiently sensitive RDTs and LM. For high-risk groups like forest workers, experts thus discuss prophylaxis or regular presumptive drug administration. Presumptive drug administration could also be considered where entire villages remain at high risk. Including 8-aminoquinolines would be an indirect way to hit the silent hypnozoite reservoir, a potentially strong blow to *P. vivax* transmission.

That hypnozoites cannot be diagnosed remains an obstacle to *P. vivax* elimination. Antibody responses to *P. vivax* antigens allowed to sensitively and specifically detect recent infections and thus hypnozoite carriage (Longley et al., 2020). Serological testing and treatment could thus become a novel tool for relapse prevention and transmission reduction.

At very low incidence, NMPs begin to follow up cases, co-exposed individuals, household members, and potentially neighbours. Spatial signatures could inform that process. Further use in similar settings must show the generalizability of distance estimates. The clustering of infections across diverse settings suggests using spatial signatures for reactive approaches also for burden reduction. The method provides a tool for NMPs to make informed decisions on operational distances, considering the number of households needed for a certain coverage vs. feasibility.



## Scientific output

### List of articles

- Sandfort, M., Vantaux, A., Kim, S., Obadia, T., Pepey, A., Gardais, S., Khim, N., Lek, D., White, M., Robinson, LJ., Witkowski, B. & Mueller, I. Forest malaria in Cambodia: the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in a cross-sectional survey in Mondulhiri province, Cambodia. *Malar J* 19, 413 (2020). <https://doi.org/10.1186/s12936-020-03482-4>
- Niang M, Sandfort M, Mbodj AF, Diouf B, Talla C, Faye J, Sane R, Thiam LG, Thiam A, Badiane A, Vigan-Womas I, Diagne N, Diene Sarr F, Mueller I, Sokhna C, White M, Toure-Balde A. Fine-scale spatio-temporal mapping of asymptomatic and clinical *P. falciparum* infections: epidemiological evidence for targeted malaria elimination interventions. *Clin Inf Dis* ciab161 (2021). <https://doi.org/10.1093/cid/ciab161>
- Sandfort M, Monteiro W, Lacerda M, Nguitragool W, Sattabongkot J, Waltmann A, Salje H, Vantaux A, Witkowski B, Robinson LJ, Mueller I, White M. The spatial signature of *Plasmodium vivax* and *P. falciparum* infections: Quantifying the clustering of infections in cross-sectional surveys and cohort studies. *In review at BMC Malaria Journal, submitted July 6<sup>th</sup> 2022. Research Square*, preprint (Version 1), 15 July 2022. <https://doi.org/10.21203/rs.3.rs-1832276/v1>
- Meyer ED\*, Sandfort M\*, Bender J\*, Matysiak-Klose D, Dörre A, Bojara G, Beyrer K, Hellenbrand W. BNT162b2 vaccination reduced infections and transmission in a COVID-19 outbreak in a nursing home in Germany, 2021. *Influenza Other Respir Viruses* Sep 9 (2022). \* joint first authors. <https://doi.org/10.1111/irv.13051>

### List of presentations

- “Accelerating towards malaria elimination in South East Asia: Identifying and delineating residual pockets of transmission”, Mirco Sandfort. Retreat of ED393 Pierre Louis de Santé Publique : Epidémiologie et Sciences de l'Information Biomédicale, Saint-Malo, 23 Oct 2017.
- “Status Report on Cross-Sectional Survey in Cambodia”, Mirco Sandfort. Asia-Pacific ICEMR SAG Meeting, Institut Pasteur Paris, 29 Jan 2018.

- “The spatial signature of *P. vivax* transmission: Insights from spatial, temporal and genetic data”, Mirco Sandfort, Ivo Mueller, Michael White. Side meeting workshop “Data analytics and modelling for *P. vivax*” at International Conference on *P. vivax* Research (ICPvR) at Institut Pasteur, Paris, June 25, 2019.
- “Spatial heterogeneity in malaria risk: Insights from cross-sectional surveys in Cambodia”, Mirco Sandfort, Amélie Vantaux, Saorin Kim, Thomas Obadia, Soazic Gardais, Nimol Khim, Michael White, Leanne Robinson, Benoit Witkowski, Ivo Mueller. International Conference on *P. vivax* Research (ICPvR) at Institut Pasteur, Paris, June 26-28, 2019.
- “Spatial heterogeneity in malaria infection risk in a remote area in Cambodia”, Mirco Sandfort. Parasites & Insect Vectors department seminar, Institut Pasteur, Paris, 16 Jan 2020.
- “Cambodian cross-sectional survey 2017-2018”, Mirco Sandfort. International Centers of Excellence for Malaria Research conference call, Institut Pasteur, Paris, 2 Apr 2020.
- As invited speaker: “Forest malaria in Cambodia: the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in a cross-sectional survey in Mondulhiri province, Cambodia.”, Sandfort, M., Vantaux, A., Kim, S., Obadia, T., Pepey, A., Gardais, S., Khim, N., Lek, D., White, M., Robinson, L.J., Witkowski, B. & Mueller, I. Joint International Tropical Medicine Meeting (JITMM) 2020, Mahidol University, Bangkok, Thailand, 15 Dec 2020.

## List of posters

- “Spatial heterogeneity and determinants of residual malaria transmission risk in North-Eastern Cambodia”, Mirco Sandfort, Thomas Obadia, Saorin Kim, Michael White, Amélie Vantaux, Benoit Witkowski, Patrice Piola, Ivo Mueller. Retreat of Parasites and Insect Vectors Department, Institut Pasteur Paris, Maintenenon, 10-12 Apr 2018.
- “Prevalence, risk heterogeneity, and spatial dependence of *P. vivax* and *P. falciparum* infections: Two cross-sectional surveys in a remote area in Cambodia offer basis to tailor malaria elimination strategies”, Mirco Sandfort, Amélie Vantaux, Saorin Kim, Thomas Obadia, Soazic Gardais, Nimol Khim, Michael White, Leanne Robinson, Benoit Witkowski, Ivo Mueller. International Conference on *P. vivax* Research (ICPvR) at Institut Pasteur, Paris, June 26-28, 2019.
- “Pockets of high malaria infection prevalence in villages inside the forest and their micro-epidemiology: A cross-sectional survey in rural Cambodia”, Mirco Sandfort, Amélie Vantaux, Saorin Kim, Thomas Obadia, Soazic Gardais, Nimol Khim, Michael White,

Leanne Robinson, Benoit Witkowski, Ivo Mueller. Retreat of ED393 Pierre Louis de Santé Publique : Epidémiologie et Sciences de l'Information Biomédicale, Saint-Malo, 21-23 Oct 2019.

- “The spatial signature of malaria infection: A new measure to calibrate transmission models and inform trial design of targeted malaria control measures”, Mirco Sandfort, Amélie Vantoux, Leanne Robinson, Benoit Witkowski, Ivo Mueller; Michael White. The Malaria Endgame, Keystone Symposium, Addis Ababa, Ethiopia, October 30-November 2, 2019.

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## **Annex**

### **Annex Chapter 3 – Supplementary material to chapter 3**

## Supplementary Material

*Table ST1 Sampling scheme across villages via linearly decreasing sampling weights with increasing village size in order to oversample smaller villages. 250 as minimal sample size if village was not smaller already.*

<b>Village</b>	<b>Size</b>	<b>Status for random selection</b>	<b>Proportion selected for random sampling</b>	<b>N finally enrolled</b>	<b>Proportion of village size enrolled</b>
Trapaingphiae	1873	Weights	49.8%	594	31.7%
Chhnaeng	1696	linearly	50.9%	550	32.4%
Oham	1364	decreasing	52.9%	333	24.4%
Ohrana	1217	with larger	53.7%	470	38.6%
Sraepreas	711	village size	56.7%	228	32.1%
Sraektum	571		57.6%	296	51.8%
Lapakhe	416	250 is more	60.1%	71	17.1%
Ohkaunpreas	383	than linearly	46.0%*	317	82.8%
Poucha	367	decreasing	68.1%	239	65.1%
Trapaingtouk	268	weights would	93.3%	201	75.0%
Sraeampilkroam	258	suggest, thus	96.9%	218	84.4%
Gaty	258	oversampled	96.9%	95	36.8%
Sraeampilleu	177	<250, thus	100%	168	94.9%
Sraelvy	159	fully sampled	100%	154	96.9%
Ohchra	157		100%	152	96.8%
Ohtrone	124		100%	103	83.1%
Beng	54		100%	11	20.4%

\* Weights and random selection were calculated based on village size of 176 identified initially during the census. Later, the field team realized to have missed a part of the village and thus appended the census.

**Table ST2** Full list of covariates and their association with prevalence (“pos.”) and risk of infection (as detected by PCR) for *P. vivax* and *P. falciparum*. Statistical significance of the covariate based on prevalence by  $\chi^2$  test (or Fisher’s exact test if required) and based on univariate logistic regression with random intercepts for households and villages by likelihood ratio test (LRT). “n.s.”: Not significant ( $p \geq 0.05$ ).

Covariate	N	<i>P. vivax</i>					<i>P. falciparum</i>				
		pos. (n)	$p(\chi^2)$	OR	95% CI	$p(LRT)$	pos. (n)	$p(\chi^2)$	OR	95% CI	$p(LRT)$
Gender	Female	2231	3.6% (80)	<0.001	Reference		1.9% (42)	<0.001	Reference		<0.001
	Male	1969	9.5% (188)		3.45	[2.60-4.57]	<0.001		4.2% (83)	2.69	
Age [years]	2-10	1054	1.5% (16)	<0.001	Reference		0.7% (7)	<0.001	Reference		<0.001
	11-15	527	7.0% (37)		4.41	[2.43-8.01]	3.0% (16)		4.44	[1.74-11.34]	
	16-20	424	11.6% (49)		8.16	[4.58-14.56]	3.8% (16)		5.25	[2.04-13.48]	
	21-25	335	10.1% (34)		8.73	[4.74-16.06]	5.4% (18)		10.18	[4.00-25.88]	
	26-30	347	10.1% (35)		9.11	[4.96-16.74]	4.9% (17)		8.49	[3.30-21.81]	
	31-35	309	8.4% (26)		6.73	[3.54-12.77]	4.5% (14)		9.21	[3.48-24.33]	
	36-40	289	9.0% (26)		6.51	[3.40-12.43]	4.2% (12)		7.48	[2.76-20.26]	
	41-45	244	6.6% (16)		4.61	[2.25-9.41]	5.3% (13)		8.66	[3.20-23.45]	
	46-50	197	6.1% (12)		4.73	[2.20-10.18]	3.6% (7)		6.40	[2.07-19.80]	

Covariate	N	<i>P. vivax</i>					<i>P. falciparum</i>					
		pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	
	51-80	474	3.6% (17)		2.88	[1.46-5.70]		1.1% (5)		1.76	[0.52-5.93]	
Forest proximity of village	Outside forest	3060	3.4% (103)		Reference			1.4% (43)		Reference		
	Forest fringe	625	7.2% (45)	<0.001	2.43	[1.03-5.73]		3.5% (22)	<0.001	2.60	[1.00-6.75]	
	Inside forest	515	23.3% (120)		9.23	[4.28-19.91]	<0.001	11.7% (60)		16.49	[6.96-39.05]	<0.001
Material of floor	Else	3578	7.0% (249)		Reference			3.2% (116)		Reference		
	Cement/tiles	622	3.1% (19)	<0.001	0.59	[0.35-0.97]	<0.05	1.4% (9)	<0.05	0.64	[0.30-1.38]	n.s.
Material of walls	No wall/tent	65	13.8% (9)		2.95	[1.29-6.76]		9.2% (6)		5.45	[1.79-16.61]	
	Wooden	3802	6.5% (248)		Reference			3.1% (116)		Reference		
	Iron sheets	217	4.6% (10)	<0.01	1.04	[0.53-2.03]	n.s.	1.4% (3)	<0.01	0.66	[0.19-2.26]	<0.01
	Cement/bricks	116	0.9% (1)		0.27	[0.04-1.72]		0% (0)		No cases		
Material of roof	Grass/leaves	81	3.7% (3)		1.54	[0.47-5.08]		1.2% (1)		1.00	[0.12-8.30]	
	Tent	79	12.7% (10)		3.93	[1.60-9.69]		2.5% (2)		1.62	[0.29-8.87]	
	Corrugated iron	3301	7.0% (232)	<0.001	Reference		<0.01	3.4% (113)	<0.01	Reference		n.s.
	Wood/cement/tiles	739	3.1% (23)		0.60	[0.38-0.96]		1.2% (9)		0.46	[0.22-1.00]	

Covariate	N	<i>P. vivax</i>					<i>P. falciparum</i>						
		pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)		
Water source	Surface/rain water	303	9.9% (30)	<0.001	1.06	[0.65-1.74]	n.s.	3.6% (11)	<0.001	0.85	[0.39-1.84]	n.s.	
	Well	2011	7.6% (152)		Reference			3.7% (74)		Reference			
	Tap	57	8.8% (5)		1.46	[0.49-4.33]		8.8% (5)		4.11	[1.09-15.48]		
	Bottled water	1828	4.4% (81)		0.79	[0.57-1.10]		1.9% (35)		0.80	[0.48-1.33]		
Toilet in house	No toilet	2383	8.2% (195)	<0.001	Reference		4.1% (97)	<0.001	Reference				
	Any type	1816	4.0% (73)		0.67	[0.47-0.94]	<0.05		1.5% (28)	0.56	[0.33-0.96]	<0.05	
Household head attended school	No	1264	8.5% (107)	<0.001	Reference		4.2% (53)	<0.01	Reference				
	Yes	2935	5.5% (161)		0.83	[0.62-1.10]	n.s.		2.5% (72)	0.70	[0.45-1.07]	n.s.	
Main income for households	Rubber plantation	95	1.1% (1)	<0.05	0.31	[0.05-2.05]	n.s.	1.1% (1)	n.s.	0.78	[0.09-6.52]	n.s.	
	Farming	3829	6.7% (256)		Reference			3.1% (120)		Reference			
	Wood logging	73	9.6% (7)		1.85	[0.79-4.34]		4.1% (3)		1.56	[0.43-5.72]		
	Driving	22	9.1% (2)		2.23	[0.45-11.08]		<0.05		4.5% (1)	1.96		[0.18-20.97]
	Workmanship	50	2.0% (1)		0.56	[0.08-3.70]		0% (0)		No cases			
	Selling/service	130	0.8% (1)		0.15	[0.02-0.94]		0% (0)		No cases			
Household owns													

Covariate		N	<i>P. vivax</i>					<i>P. falciparum</i>				
			pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)
...chicken	No	1088	5.0% (54)	<0.05	Reference			2.5% (27)	n.s.	Reference		
	Yes	3111	6.9% (214)		1.12	[0.80-1.57]	n.s.	3.2% (98)		1.04	[0.63-1.73]	n.s.
...dogs	No	1585	4.9% (77)	<0.01	Reference			2.5% (39)	n.s.	Reference		
	Yes	2614	7.3% (191)		1.25	[0.92-1.70]	n.s.	3.3% (86)		1.02	[0.64-1.61]	n.s.
...pigs	No	3107	4.0% (123)	<0.001	Reference			1.7% (54)	<0.001	Reference		
	Yes	1092	13.3% (145)		1.40	[0.98-1.99]	n.s.	6.5% (71)		1.53	[0.89-2.61]	n.s.
...cows	No	3451	5.0% (172)	<0.001	Reference			2.2% (77)	<0.001	Reference		
	Yes	748	12.8% (96)		1.45	[1.02-2.06]	<0.05	6.4% (48)		1.61	[0.97-2.68]	n.s.
...buffaloes	No	3977	5.9% (234)	<0.001	Reference			2.7% (109)	<0.001	Reference		
	Yes	222	15.3% (34)		1.90	[1.12-3.20]	<0.05	7.2% (16)		1.98	[0.94-4.16]	n.s.
...ducks	No	3938	6.6% (259)	<0.10	Reference			2.9% (115)	n.s.	Reference		
	Yes	261	3.4% (9)		0.80	[0.40-1.61]	n.s.	3.8% (10)		1.98	[0.90-4.37]	n.s.
...cats	No	3687	6.6% (244)	n.s.	Reference			3.2% (118)	<0.05	Reference		
	Yes	512	4.7% (24)		0.83	[0.49-1.39]	n.s.	1.4% (7)		0.58	[0.24-1.40]	n.s.



Covariate	N	<i>P. vivax</i>					<i>P. falciparum</i>					
		pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	
Household head had received information on malaria in the past 3 months via ...TV	No	3784	6.9% (262)	<0.001	Reference		3.2% (122)	<0.01	Reference			
	Yes	415	1.4% (6)		0.31	[0.14-0.70]	<0.01		0.7% (3)	0.39	[0.11-1.32]	n.s.
...household visit	No	3983	6.2% (248)	n.s.	Reference		2.9% (114)	<0.10	Reference			
	Yes	216	9.3% (20)		0.76	[0.42-1.38]	n.s.		5.1% (11)	1.20	[0.54-2.64]	n.s.
Insecticides had been sprayed inside the house in the past year	No	2935	7.3% (214)	<0.001	Reference		3.6% (106)	<0.001	Reference			
	Yes	1264	4.3% (54)		0.60	[0.43-0.84]	<0.01		1.5% (19)	0.40	[0.23-0.71]	<0.001
Work-unrelated travels overnight in the last month to...	None	3465	4.9% (171)	<0.001	Reference		2.0% (70)	<0.001	Reference			
	Field sites	90	3.3% (3)		0.81	[0.26-2.51]	<0.001		4.4% (4)	2.61	[0.82-8.36]	<0.001
	Forest sites	268	22.4% (60)		5.47	[3.73-8.01]			14.9% (40)	8.38	[4.96-14.18]	
	A village	177	13.6% (24)		2.79	[1.68-4.61]			5.6% (10)	2.37	[1.08-5.17]	
	A city	59	1.7% (1)		0.44	[0.07-2.85]	0% (0)		No cases			
Unspecified	123	7.3% (9)	2.14	[1.03-4.42]	0.8% (1)	0.50	[0.06-3.94]					

Covariate	N	<i>P. vivax</i>					<i>P. falciparum</i>				
		pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)
Work in the last two months in... ...nearby forest	No	4053	6.2% (252)	Reference			2.9% (116)		Reference		
	Yes	147	10.9% (16)	<0.05	1.37	[0.77-2.44]	n.s.	6.1% (9)	<0.05	1.42	[0.64-3.15]
...deep forest	No	4055	5.7% (231)	Reference			2.4% (98)		Reference		
	Yes	145	25.5% (37)	<0.001	8.03	[4.83-13.35]	<0.001	18.6% (27)	<0.001	10.75	[5.81-19.89]
...rubber plantations	No	3904	6.8% (264)	Reference			3.2% (124)		Reference		
	Yes	296	1.4% (4)	<0.001	0.60	[0.22-1.61]	n.s.	0.3% (1)	<0.01	0.26	[0.03-2.14]
...cashew nut plantations	No	3053	5.2% (158)	Reference			2.5% (77)		Reference		
	Yes	1147	9.6% (110)	<0.001	1.10	[0.82-1.48]	n.s.	4.2% (48)	<0.01	0.90	[0.58-1.40]
...cassava field	No	2115	5.6% (118)	Reference			2.9% (62)		Reference		
	Yes	2085	7.2% (150)	<0.05	1.71	[1.28-2.28]	<0.001	3.0% (63)	n.s.	1.61	[1.03-2.50]
...rice field	No	3872	6.1% (238)	Reference			2.7% (106)		Reference		
	Yes	328	9.1% (30)	<0.05	0.75	[0.48-1.19]	n.s.	5.8% (19)	<0.01	1.19	[0.65-2.19]
No work	No	3186	7.9% (253)	Reference			3.7% (119)		Reference		
	Yes	1014	1.5% (15)	<0.001	0.24	[0.14-0.40]	<0.001	0.6% (6)	<0.001	0.23	[0.10-0.53]

Covariate		N	<i>P. vivax</i>					<i>P. falciparum</i>				
			pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)	pos. (n)	<i>p</i> ( $\chi^2$ )	OR	95% CI	<i>p</i> (LRT)
Having slept outside last night	Indoors	4124	6.2% (256)	<0.01	Reference			2.9% (118)	<0.01	Reference		
	Outdoors	76	15.8% (12)		3.11	[1.54-6.30]	<0.01	9.2% (7)		2.97	[1.14-7.74]	<0.05
Sprays repellent usually at bedtime	No	3668	6.7% (247)	<0.05	Reference			3.2% (116)	<0.10	Reference		
	Yes	524	4.0% (21)		0.62	[0.38-1.01]	n.s.	1.7% (9)		0.60	[0.28-1.27]	n.s.
Slept under net last night	No	320	10.0% (32)	<0.01	Reference			6.6% (21)	<0.001	Reference		
	Yes	3880	6.1% (236)		0.46	[0.30-0.71]	<0.01	2.7% (104)		0.32	[0.18-0.56]	<0.001
Antimalarials in the last 2 months	No	3978	6.0% (238)	<0.001	Reference			2.3% (91)	<0.001	Reference		
	Yes	211	14.2% (30)		1.73	[1.10-2.73]	<0.05	16.1% (34)		5.98	[3.52-10.15]	<0.001
Felt sick at interview	No	4017	6.4% (258)	n.s.	Reference			2.8% (114)	<0.05	Reference		
	Yes	170	5.9% (10)		0.98	[0.51-1.91]	n.s.	6.5% (11)		2.47	[1.19-5.14]	<0.05
Felt feverish at interview	No	3930	6.4% (252)	n.s.	Reference			2.7% (108)	<0.001	Reference		
	Yes	249	6.4% (16)		0.88	[0.51-1.51]	n.s.	6.8% (17)		2.39	[1.31-4.38]	<0.01
Axillary temperature	<37.5°C	3917	6.4% (250)	n.s.	Reference			2.7% (106)	<0.001	Reference		
	≥37.5°C	273	5.5% (15)		0.72	[0.41-1.24]	n.s.	7.0% (19)		2.40	[1.34-4.28]	<0.01

**Table ST3** Fixed effects parameters of multivariate logistic regression model for *Plasmodium* spp. infection as detected by PCR, including a gender-age interaction term.

Covariate		$\beta$	Std. error	<i>p</i>
(Intercept)		-4.67888	0.46687	
Gender	Female	Reference		
	Male	-0.47247	0.46214	
Age [years] alone	2-10	Reference		
	11-15	1.06923	0.40483	
	16-20	1.23856	0.40158	
	21-25	0.60961	0.49079	
	26-30	0.46972	0.50472	
	31-35	0.91935	0.49992	
	36-40	0.32925	0.51226	
	41-45	0.81629	0.52053	
	46-50	0.57800	0.63309	<0.001
	51-80	-0.19417	0.57775	
Age [years] in men (gender-age interaction)	2-10	Reference		
	11-15	0.68542	0.58371	
	16-20	1.31719	0.58402	
	21-25	2.43349	0.65292	
	26-30	2.63857	0.66262	
	31-35	1.92763	0.66384	
	36-40	2.74284	0.67905	
	41-45	1.48228	0.70859	
	46-50	1.47956	0.81076	
	51-80	1.92305	0.73188	
Forest proximity of village	Outside forest	Reference		
	Forest fringe	0.77026	0.39881	<0.001
	Inside forest	2.59209	0.36768	
Material of roof	Grass/leaves	-0.03273	0.67613	
	Tent	1.35235	0.48477	
	Corrugated iron	Reference		<0.01
	Wood planks/ cement/tiles	-0.70368	0.26758	
Household owns a toilet	No	Reference		0.06
	Yes	-0.35946	0.18914	
Household owns buffaloes	No	Reference		<0.01
	Yes	0.82853	0.28977	
Household head had received information	No	Reference		<0.01
	Yes	-1.02219	0.40875	

on malaria  
via TV in the  
past 3  
months

Insecticides had been sprayed inside the house in the past year	No Yes	Reference -0.47608	0.18622	<0.01
Having slept outside last night	No Yes	Reference 0.72020	0.39234	0.07
Having slept under a net last night	No Yes	Reference -0.01898	0.24718	0.94
Work- unrelated travels overnight in the last month to...	None Field sites Forest sites A village A city Unspecified	Reference -0.52234 0.68471 0.14994 -1.42758 0.12539	0.48253 0.22425 0.27518 1.06795 0.41792	<0.05
Work in the last two months...				
...in the deep forest	No Yes	Reference 0.95113	0.27823	<0.001
...in a cassava field	No Yes	Reference 0.31620	0.15641	<0.05

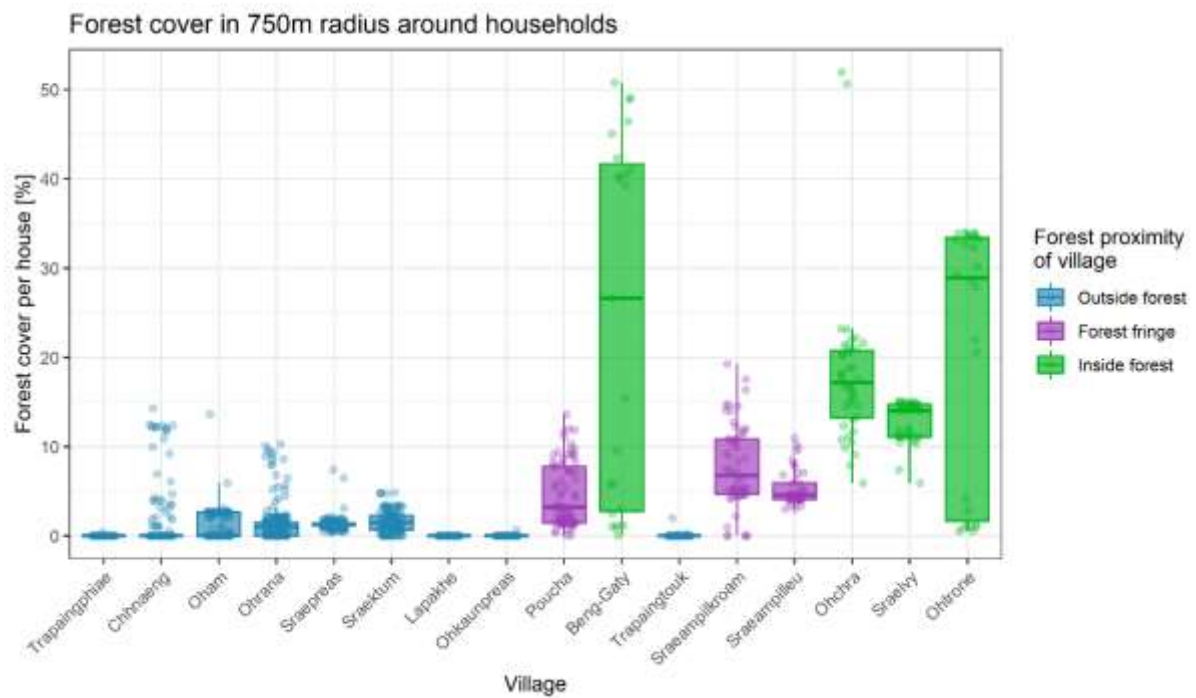
**Table ST4** Details of spatial clusters identified by covariate-adjusted SaTScan analysis.

Cluster	Lat	Long	Radius	# Pop	# Obs	# Exp	RR	<i>p</i>
1	12.22155	106.9107	4.5 km	273	80	37	2.5	<0.001
2	12.24500	106.8488	2.5 km	152	60	28	2.4	<0.001

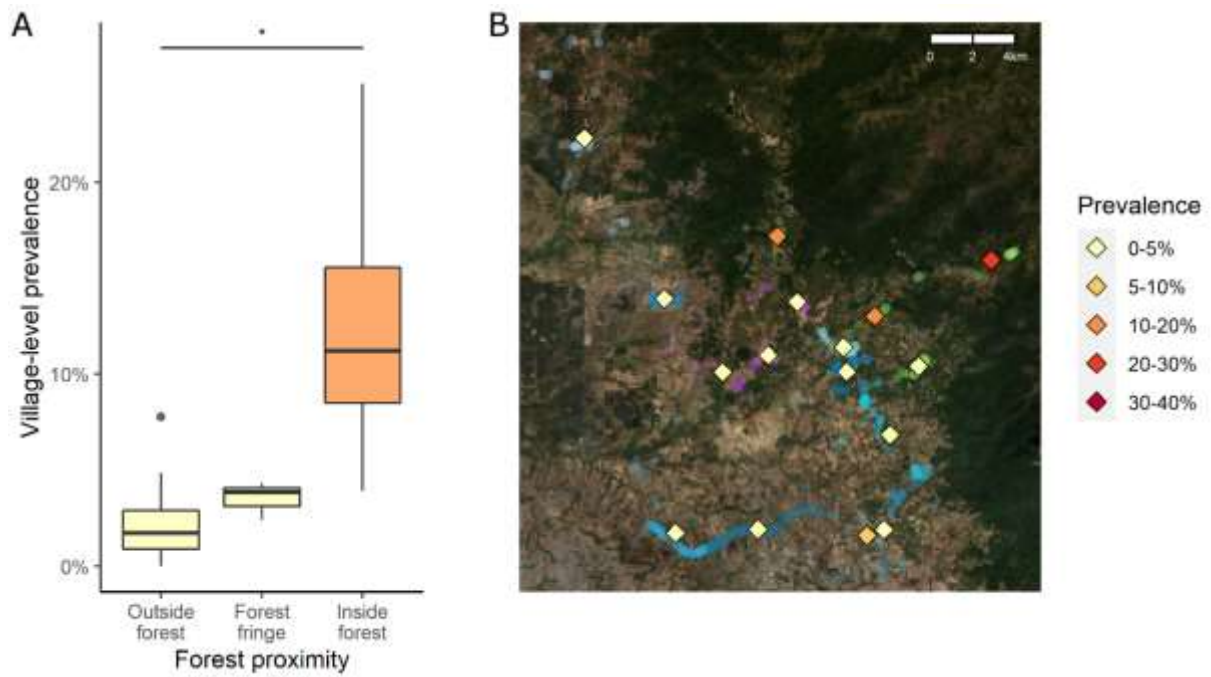
**Table ST5** Portion of villages that form the spatial clusters in the SaTScan analysis.

Village	Cluster	# households	# households in cluster	% of households in cluster
Beng-Gaty	1	42	37	88.1%
Sraelvy	1	36	20	55.6%
Ohtrone	1	23	19	82.6%
Ohchra	2	39	39	100%

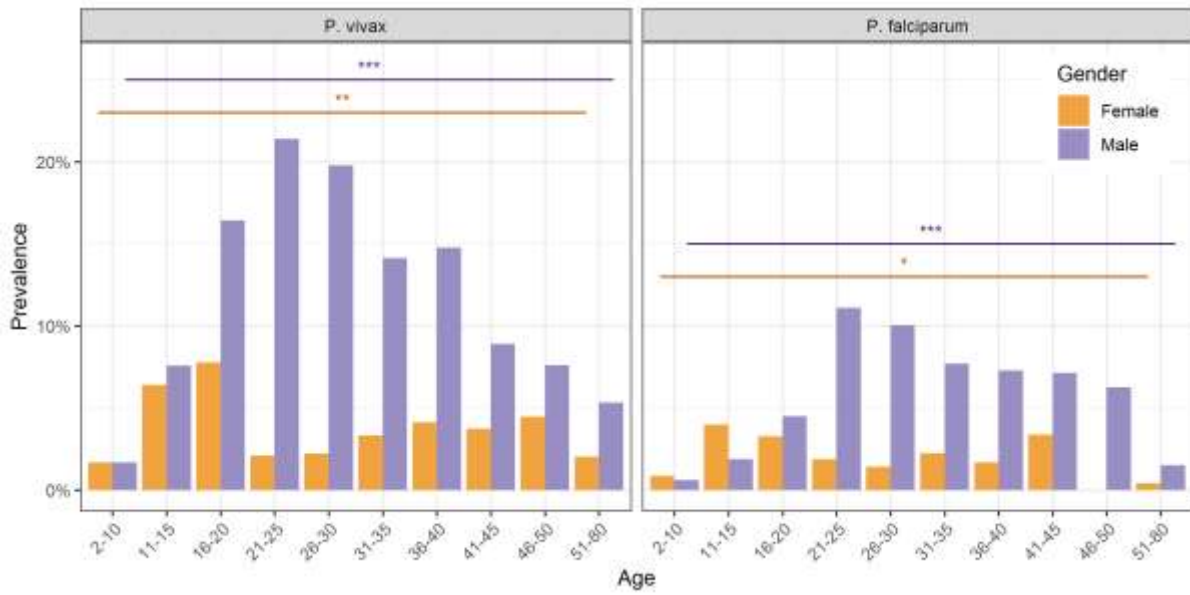
## Supplementary Figures



**Figure S1** Classification of villages as to their forest proximity based on forest cover in 750 m vicinity around census households. Forest cover was calculated by remote sensing from satellite imagery as in [1].



**Figure S2** Prevalence of *P. falciparum* infection per village (as boxplots by proximity of villages to the forest in panel A and as squares in panel B). Household locations of survey participants in the map background, transparently coloured by village in shades of blue, purple, or green if village is in category “outside forest”, “forest fringe”, or “inside forest”, resp. Significance asterisk for Kruskal-Wallis test of differences in village-level prevalence.



**Figure S3** Prevalence of infection by age per gender and species. Significance asterisks for test of differences in prevalence across age groups per gender strata.

## References

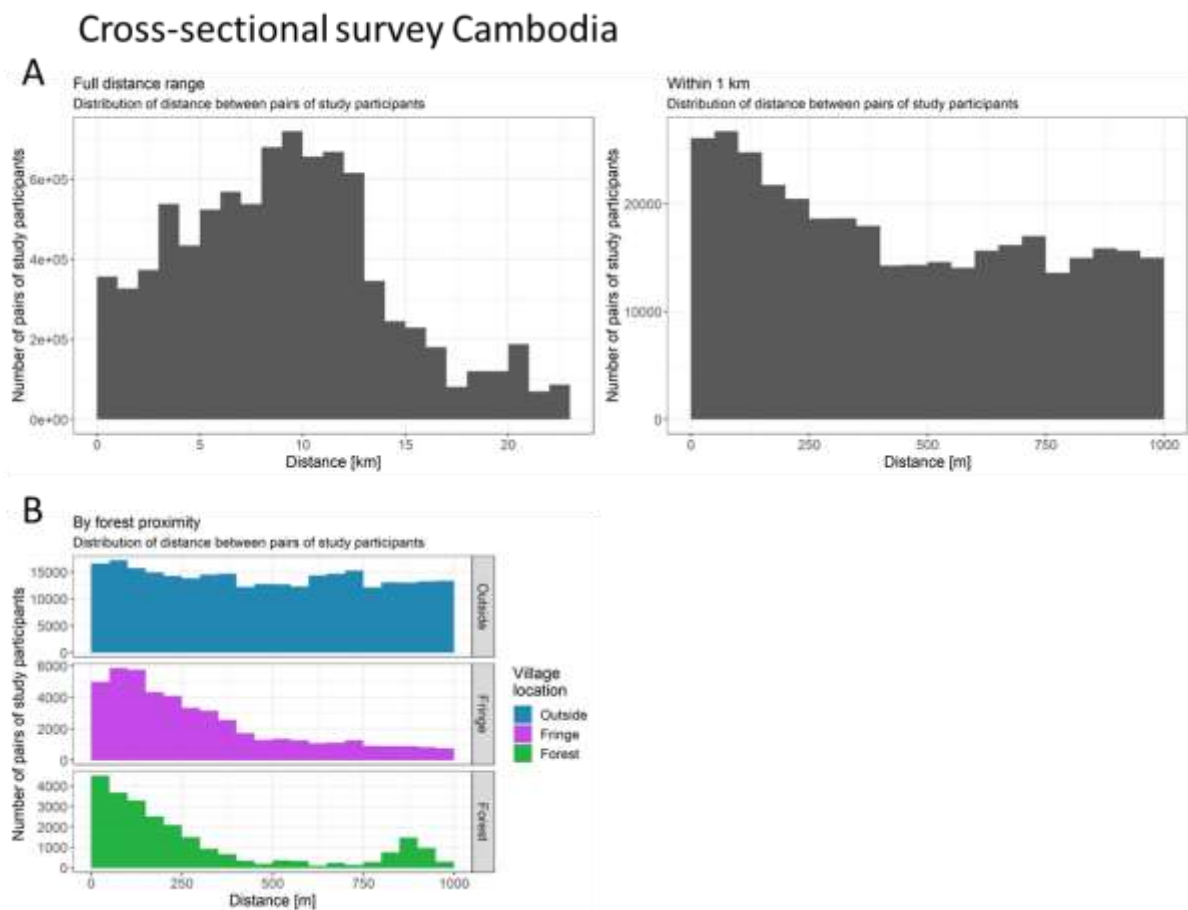
1. Pepey A, Souris M, Vantaux A, Morand S, Lek D, Mueller I, et al. Studying Land Cover Changes in a Malaria-Endemic Cambodian District: Considerations and Constraints. *Remote Sens.* 2020;12:2972.



**Annex Chapter 4 – Supplementary material to chapter 4**

1 Supplementary Material -  
2 The spatial signature of *Plasmodium vivax* and *P. falciparum*  
3 infections: Quantifying the clustering of infections in cross-  
4 sectional surveys and cohort studies  
5 Mirco Sandfort, Wuelton Monteiro, Marcus Lacerda, Wang Nguitrangool, Jetsumon Sattabongkot,  
6 Andreea Waltmann, Henrik Salje, Amélie Vantaux, Benoit Witkowski, Leanne J. Robinson, Ivo  
7 Mueller, Michael White

8

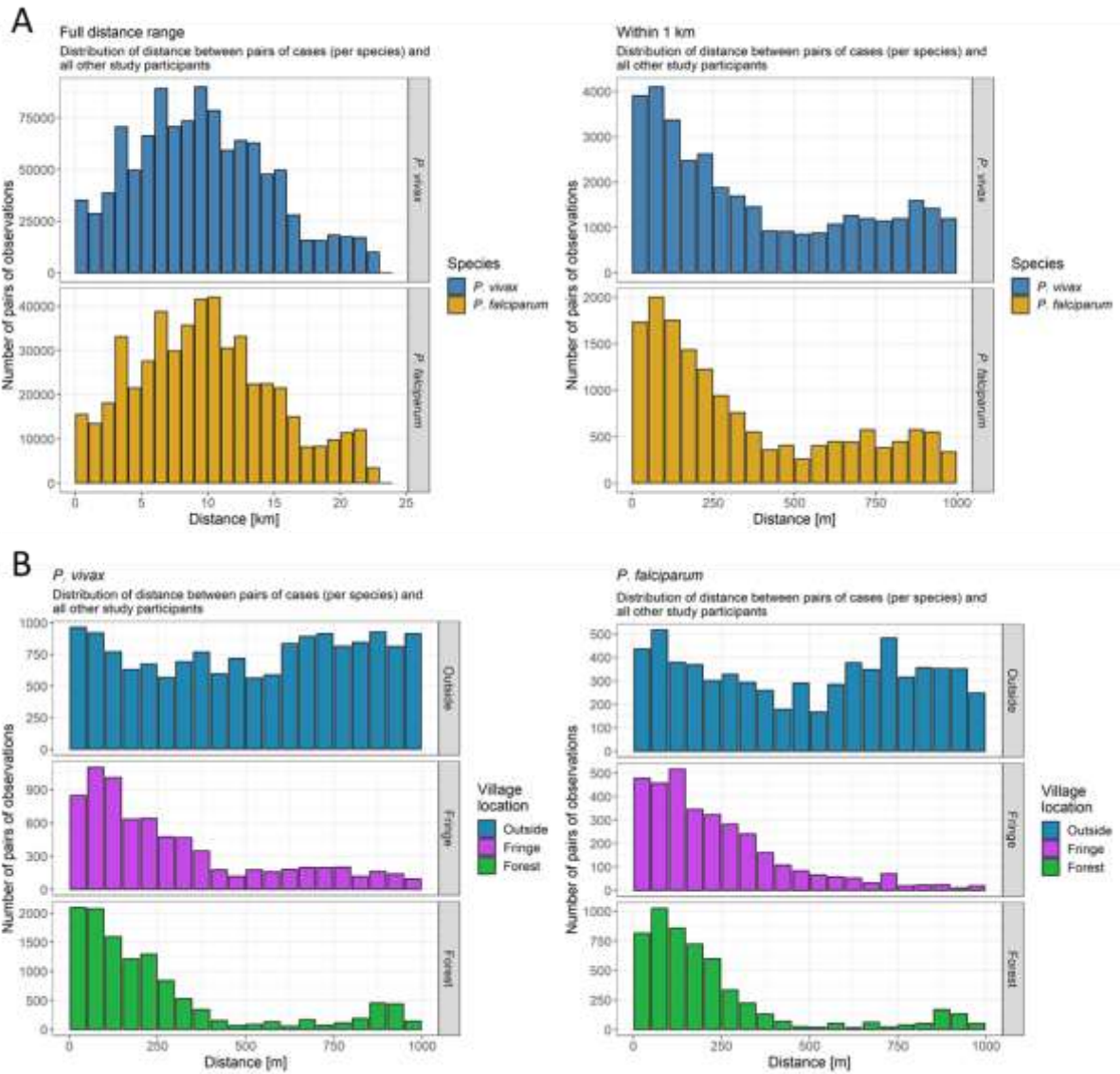


9

10 Fig. S1.1a Distribution of distance between pairs of study participants globally (panel A, left) and  
11 within 1 km (right) and stratified by the villages' proximity to the forest (panel B) for the cross-  
12 sectional survey in Cambodia.

13

# Cross-sectional survey Cambodia

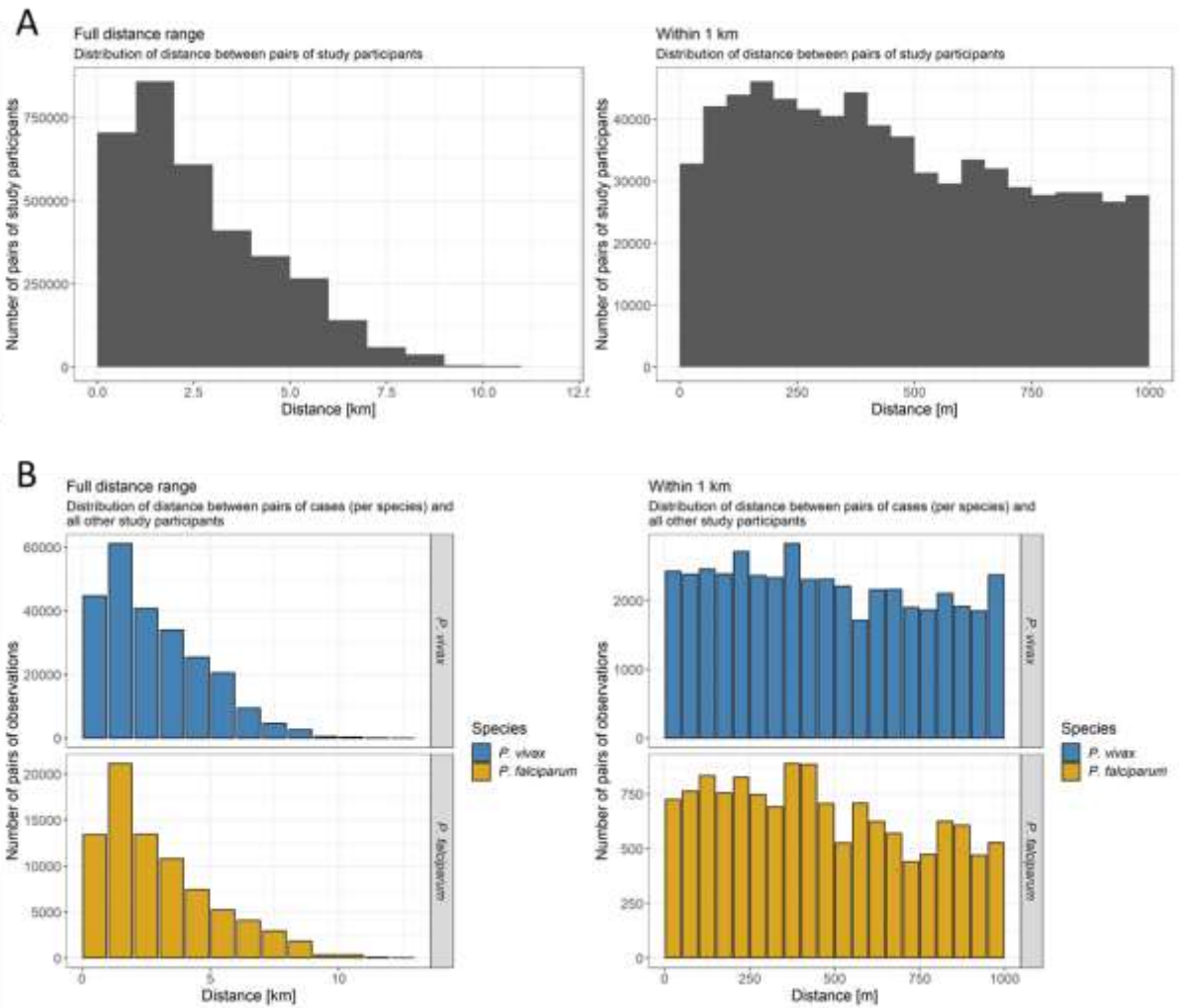


14

15 Fig. S1.1b Distribution of distance between pairs of cases and all other study participants globally  
 16 (panel A, left) and within 1 km (right) per species for the cross-sectional survey in Cambodia.  
 17 Stratified by the villages' proximity to the forest (panel B).

18

# Cross-sectional survey Thailand

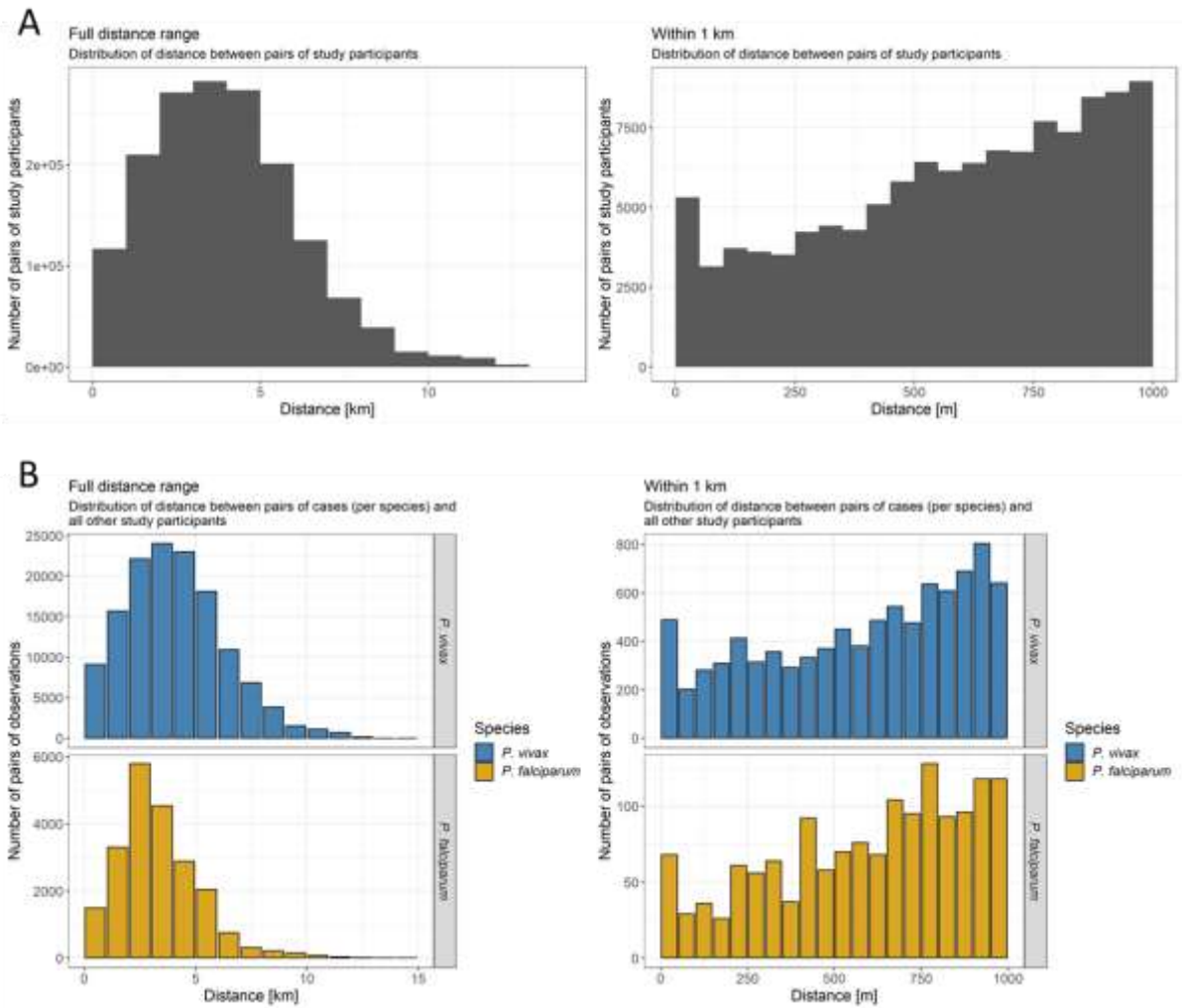


19

20 Fig. S1.2 Distribution of distance between pairs of study participants globally (panel A, left) and  
 21 within 1 km (right) for the cross-sectional survey in Thailand. Distribution of distance between pairs  
 22 of cases and all other study participants globally (panel B, left) and within 1 km (right) per species.

23

## Cross-sectional survey 1 Brazil

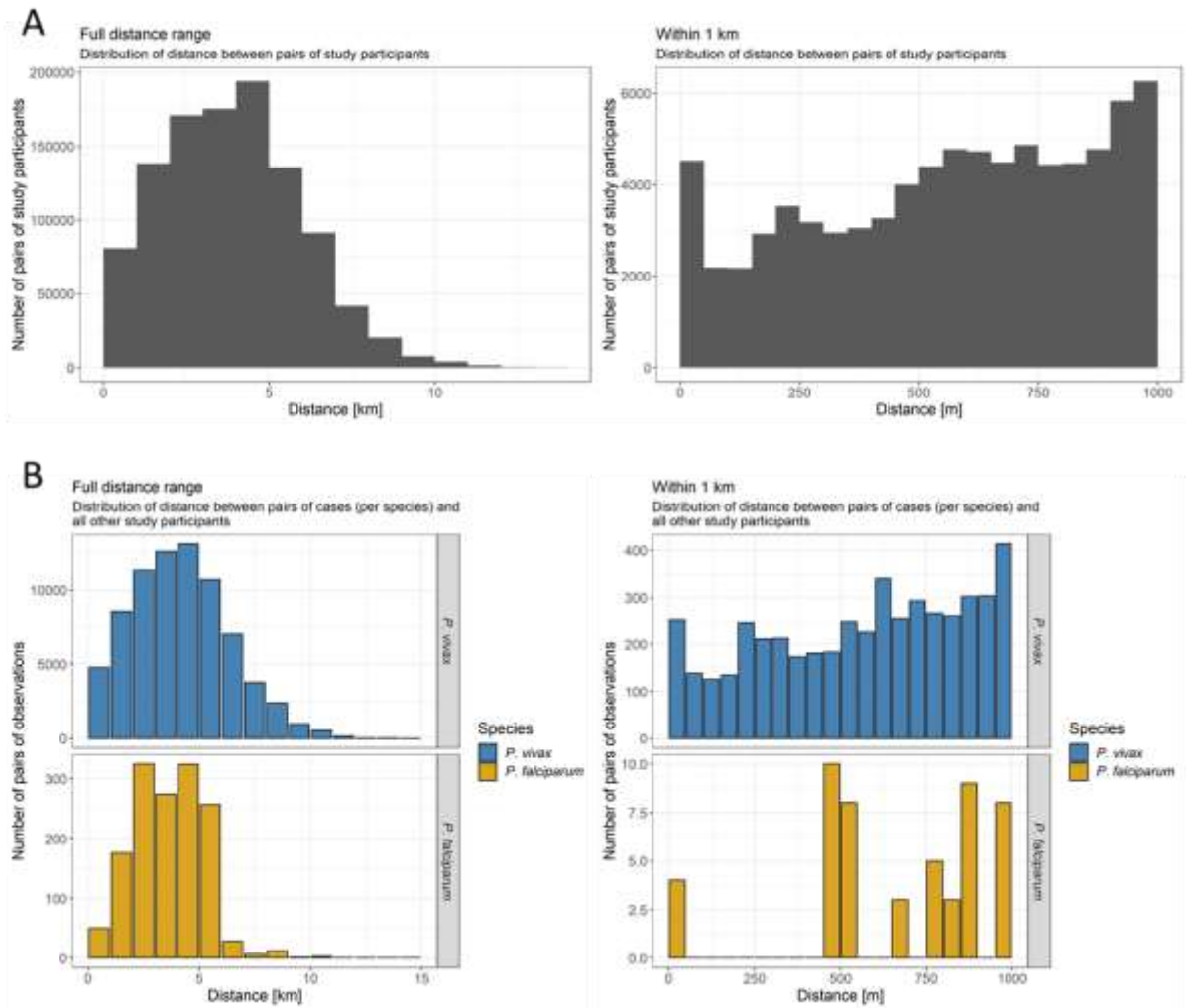


24

25 Fig. S1.3 Distribution of distance between pairs of study participants globally (panel A, left) and  
 26 within 1 km (right) for the first cross-sectional survey in Brazil. Distribution of distance between pairs  
 27 of cases and all other study participants globally (panel B, left) and within 1 km (right) per species.

28

## Cross-sectional survey 2 Brazil

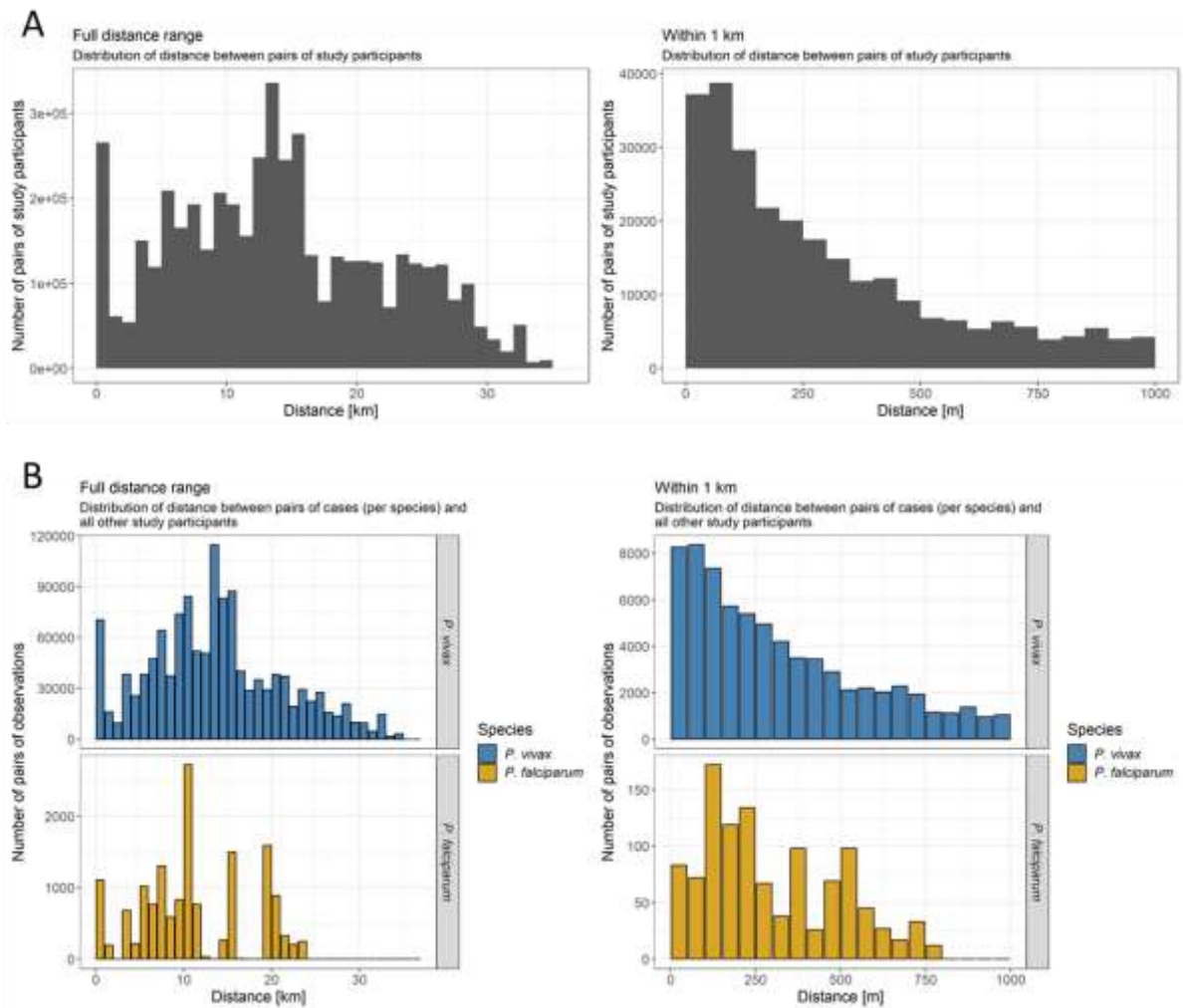


29

30 Fig. S1.4 Distribution of distance between pairs of study participants globally (panel A, left) and  
 31 within 1 km (right) for the second cross-sectional survey in Brazil. Distribution of distance between  
 32 pairs of cases and all other study participants globally (panel B, left) and within 1 km (right) per  
 33 species.

34

## Cross-sectional survey Solomon Islands

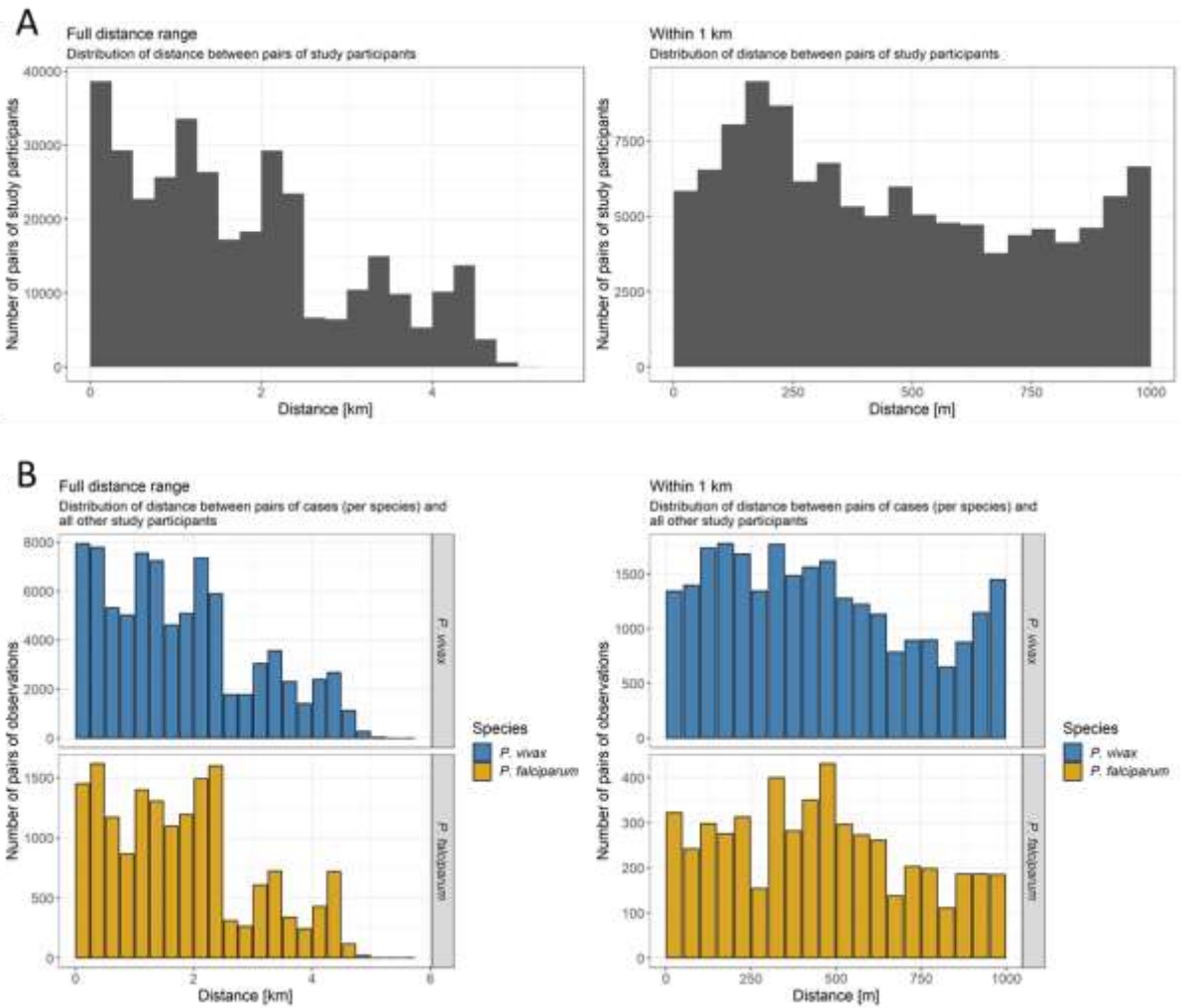


35

36 Fig. S1.5 Distribution of distance between pairs of study participants globally (panel A, left) and  
 37 within 1 km (right) for the cross-sectional survey in Solomon Islands. Distribution of distance  
 38 between pairs of cases and all other study participants globally (panel B, left) and within 1 km (right)  
 39 per species.

40

# Cohort study Thailand



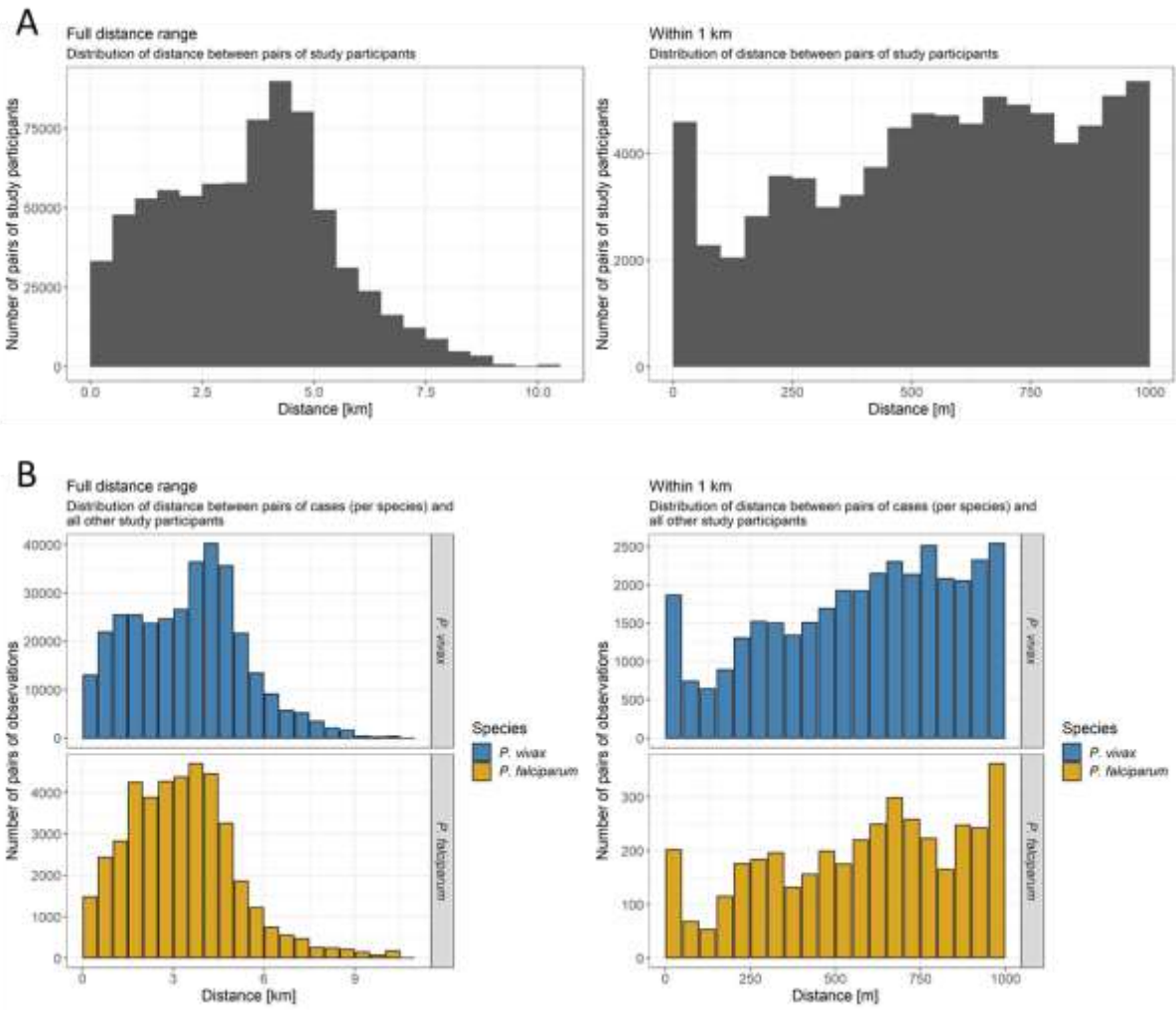
41

42 Fig. S1.6 Distribution of distance between pairs of study participants globally (panel A, left) and  
 43 within 1 km (right) for the cohort study in Thailand. Distribution of distance between pairs of cases  
 44 and all other study participants globally (panel B, left) and within 1 km (right) per species.

45



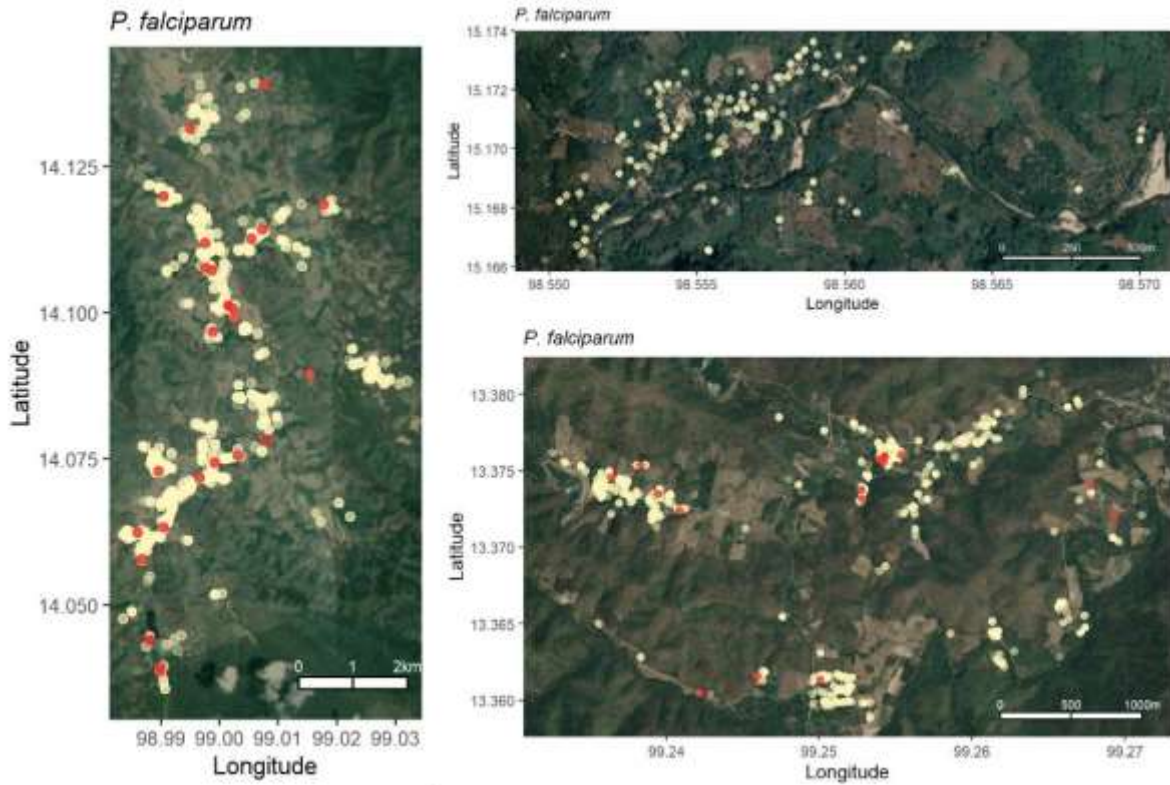
# Cohort study Brazil



46

47 Fig. S1.7 Distribution of distance between pairs of study participants globally (panel A, left) and  
 48 within 1 km (right) for the cohort study in Brazil. Distribution of distance between pairs of cases and  
 49 all other study participants globally (panel B, left) and within 1 km (right) per species.

50



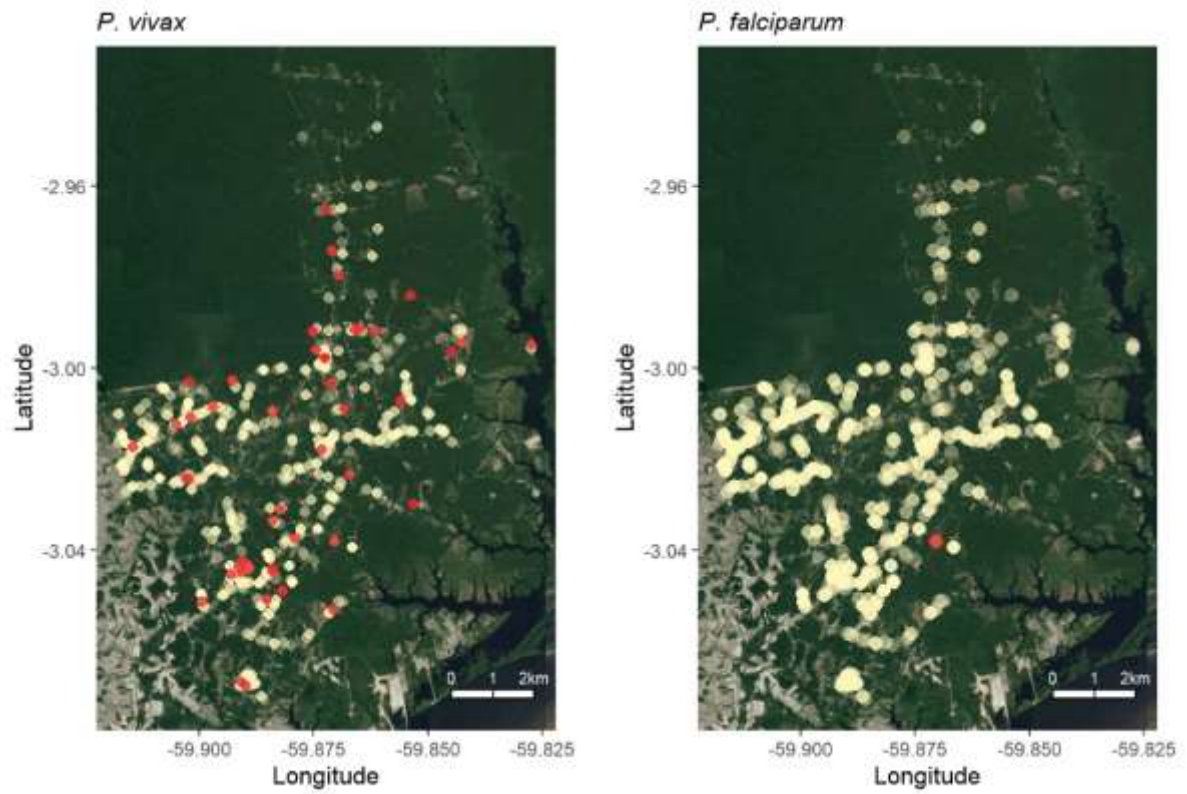
51

52 Fig. S2 Locations of households and *P. falciparum* infections in the cross-sectional survey in Thailand.

53 Central region left panel, Northern region upper right panel, Southern region lower right panel.

54 Household locations in light gold. Infections in red.

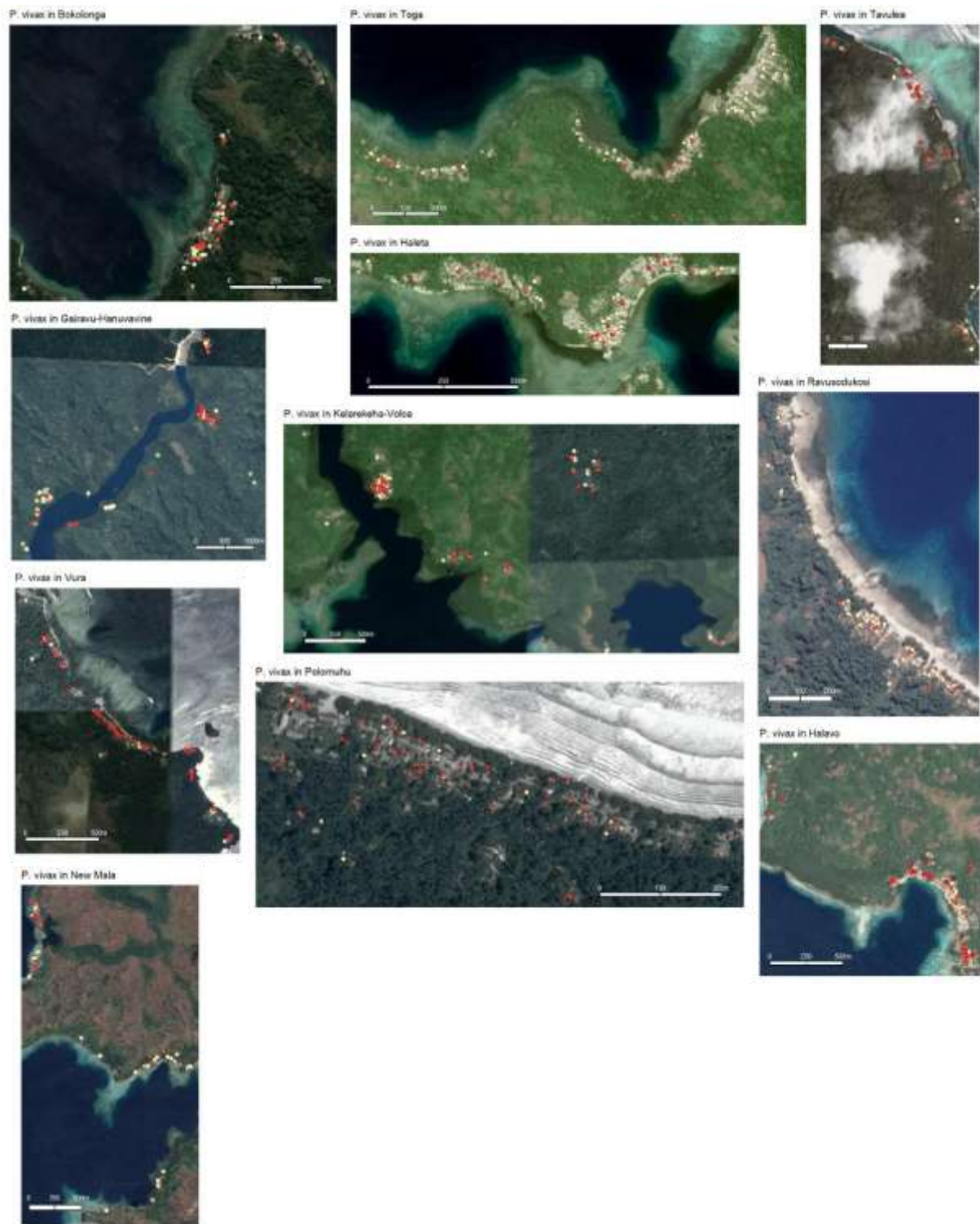
55



56

57 Fig. S3 Locations of households and *P. vivax* and *P. falciparum* infections in the second cross-sectional  
58 survey in Brazil. Household locations in light gold. Infections in red.

59



60

61 Fig. S4a Locations of households and *P. vivax* infections in the villages of the cross-sectional survey in  
 62 Solomon Islands (not part of Figure 2 in the main text). Household locations in light gold. Infections in  
 63 red.

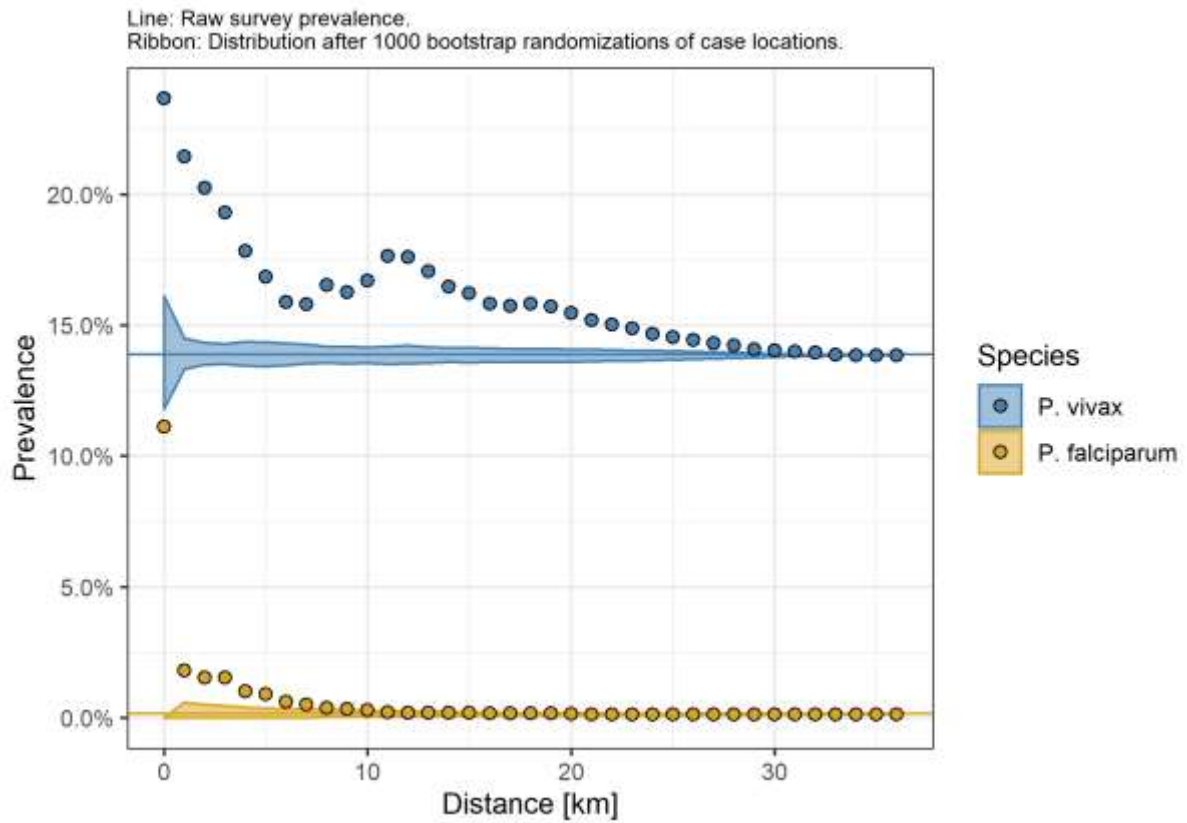
64



65

66 Fig. S4b Locations of households and *P. falciparum* infections in the villages of the cross-sectional  
 67 survey in Solomon Islands. Household locations in light gold. Infections in red.

68



69

70 Fig. S5 The spatial signature of prevalence of *P. vivax* or *P. falciparum* infections in the cross-sectional  
 71 survey in Solomon Islands across the full distance range. Ribbon: 95%-quantile interval of null  
 72 distribution. Horizontal line: Global survey prevalence.

73

**Annex Chapter 5 – Supplementary material to chapter 5**

**Table S1: Individual vs household risk analysis of the relationship between asymptomatic *P. falciparum* carriage and subsequent clinical malaria episode in Dielmo village..**

<b>Analysis at individual level</b>						
	<b>2013</b>	<b>2014</b>	<b>2015</b>	<b>2016</b>	<b>All years</b>	
<b>PCR prevalence</b>	4.3% (13/301)	12.4% (43/348)	6.7% (24/357)	6.2% (22/354)	7.5% (102/1360)	
<b>incidence of clinical malaria</b>	14.4% (45/312)	13.9% (49/353)	0.8% (3/357)	0.8% (3/356)	7.3% (100/1378)	
<b>PCR status at cross-section</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>
<b>proportion with clinical episode</b>	11.1% (32/288)	15.4% (2/13)	12.1% (37/305)	16.3% (7/43)	0.9% (3/333)	0% (0/24)
<b>P value for difference in proportions</b>	<b>P = 0.65</b>	<b>P = 0.46</b>	<b>P = 1</b>	<b>P = 1</b>	<b>P = 1</b>	<b>P = 0.20</b>
<b>Analysis at household level</b>						
<b>PCR prevalence</b>	16.7% (7/42)	50.0% (22/44)	31.1% (14/45)	31.2% (15/48)	32.4% (58/179)	
<b>incidence of clinical malaria</b>	42.9% (18/42)	47.7% (21/44)	6.7% (3/45)	6.3% (3/48)	25.1% (45/179)	
<b>PCR status at cross-section</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>
<b>proportion with clinical episode</b>	34.3% (12/35)	85.7% (6/7)	18.2% (4/22)	77.3% (17/22)	0.0% (0/31)	21.4% (3/14)
<b>P value for difference in proportions</b>	<b>P = 0.031*</b>	<b>P = 0.0002*</b>	<b>P = 0.026*</b>	<b>P = 0.23</b>	<b>P = 2 x 10<sup>-6</sup>*</b>	<b>P = 2 x 10<sup>-6</sup>*</b>

Fisher's exact test was applied to statistical difference between proportions

\* Denotes statistical significance at 5% level



**Table S2: Individual vs household risk analysis of the relationship between asymptomatic *P. falciparum* carriage and subsequent clinical malaria episode in Ndiop village.**

<b>Analysis at individual level</b>						
	<b>2013</b>	<b>2014</b>	<b>2015</b>	<b>2016</b>	<b>All years</b>	
<b>PCR prevalence</b>	2.6% (8/310)	12.9% (47/365)	5.2% (20/384)	17.2% (64/373)	9.7% (139/1432)	
<b>incidence of clinical malaria</b>	24.4% (81/332)	10.1% (37/366)	2.6% (10/385)	2.1% (8/376)	9.3% (136/1459)	
<b>PCR status at cross-section</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>
<b>proportion with clinical episode</b>	19.2% (58/302)	12.5% (1/8)	8.5% (27/318)	19.1% (9/47)	0% (0/20)	1.6% (1/64)
<b>P value for difference in proportions</b>	<b>P = 1</b>	<b>P = 0.033*</b>	<b>P = 1</b>	<b>P = 1</b>	<b>P = 0.87</b>	<b>P = 0.87</b>
<b>Analysis at household level</b>						
	<b>2013</b>	<b>2014</b>	<b>2015</b>	<b>2016</b>	<b>All years</b>	
<b>PCR prevalence</b>	18.8% (6/32)	62.5% (20/32)	40.0% (14/35)	35.1% (13/37)	39.0% (53/136)	
<b>incidence of clinical malaria</b>	65.6% (21/32)	37.5% (12/32)	11.4% (4/35)	16.2% (6/37)	31.6% (43/136)	
<b>PCR status at cross-section</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>	<b>PCR -ve</b>	<b>PCR +ve</b>
<b>proportion with clinical episode</b>	57.7% (15/26)	100% (6/6)	16.7% (2/12)	50.0% (10/20)	4.2% (1/24)	38.5% (5/13)
<b>P value for difference in proportions</b>	<b>P = 0.07</b>	<b>P = 0.08</b>	<b>P = 0.28</b>	<b>P = 0.014*</b>	<b>P = 0.008*</b>	<b>P = 0.008*</b>

Fisher's exact test was applied to statistical difference between proportions

\* Denotes statistical significance at 5% level

## **Detection and characterization of *Plasmodium* species by LM and qPCR**

### **Microscopic examination**

Thick and thin blood films were prepared, stained with 10% Giemsa for 25 minutes and screened for the presence of malaria parasites using LM with 100x oil immersion lens. The number of parasites per 200 white blood cells in thick film was recorded and parasite density was estimated by counting the number of leucocytes by field examined and by arbitrarily considering that 8,000 leucocytes were present in 1 $\mu$ l of blood. Two microscopists read all slides, and in case of discrepancy, a third microscopist examined the slides and the results were combined. A slide was considered positive after two concordant readings and at least, two hundred oil-immersion fields were examined before a slide was declared negative for *Plasmodium*.

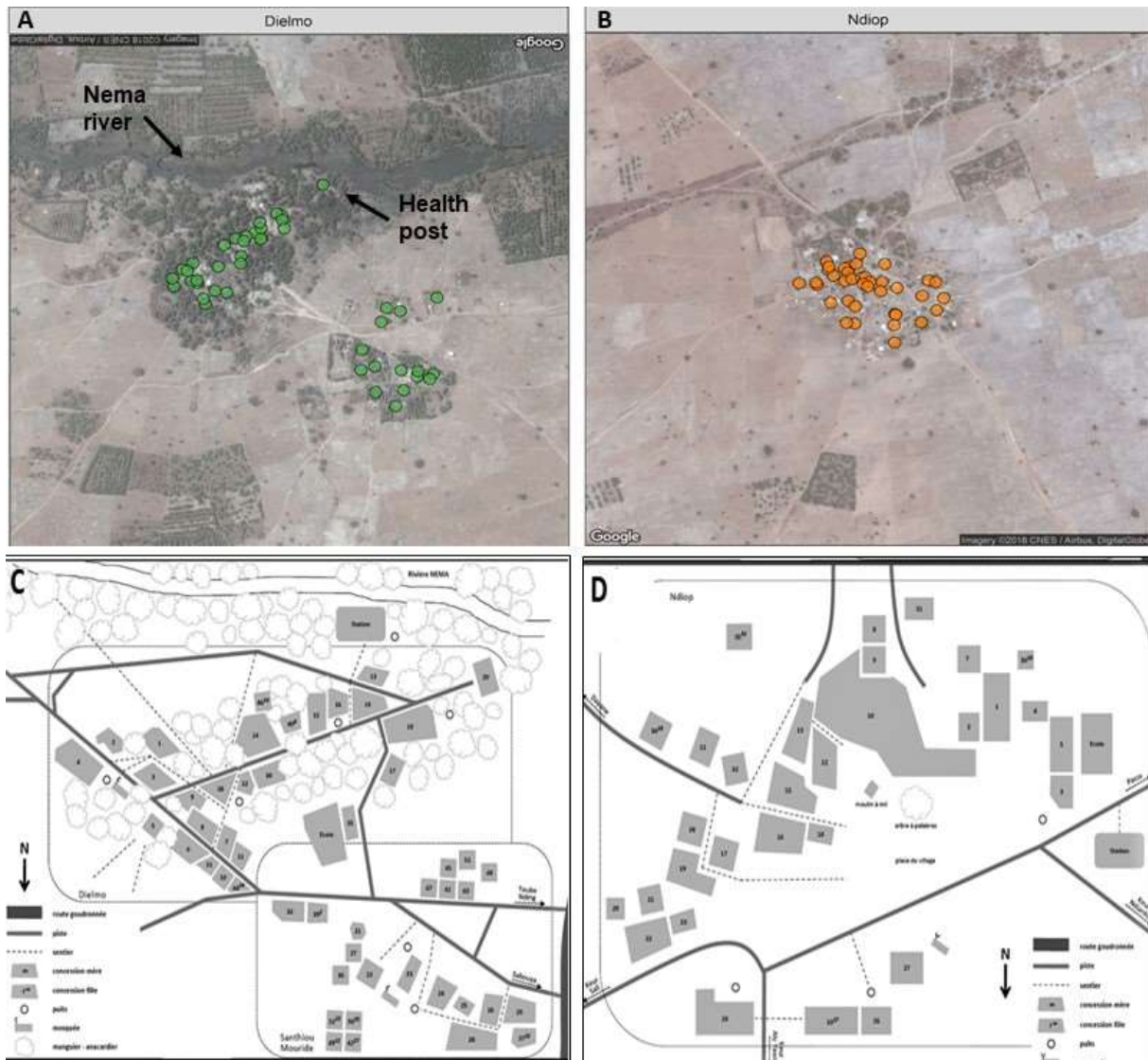
### **Molecular detection and characterization of *Plasmodium* species**

The detection of *Plasmodium* spp was carried out by qPCR following genomic DNA isolation of *Plasmodium* parasites using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Genomic DNA from blood samples of microscopically-confirmed *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* infected patients served for positive controls in all amplifications; sterile water and gDNA from uninfected blood samples served for negative controls to ensure lack of contamination [20].

The qPCR-based detection and characterization of *Plasmodium* spp was based on two steps real time PCR as previously described [21, 22]. Firstly, *Plasmodium* parasites were detected by a "screening real-time PCR" with genus-specific primers targeting the *Plasmodium cytochrome b* gene using the Eva Green dye (Solis Biodyne) followed by melt curve analysis of resulting amplicons from the Bio-Rad CFX96 real time thermal cycler as described previously [21]. Secondly, positive *Plasmodium* DNA samples were ten-fold diluted and analyzed for malaria species using a nested real-time PCR assays with genus-specific primers

for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* as described by Canier et al. [21]. The presence of *P. knowlesi*, the fifth human malaria parasite [23] was not investigated in this study.

### Supplementary Figure S1



Supplementary Figure S2

