High Genetic Diversity of *Plasmodium vivax* on the North Coast of Papua New Guinea

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Abstract. Despite having the highest *Plasmodium vivax* burden in the world, molecular epidemiological data from Papua New Guinea (PNG) for this parasite remain limited. To investigate the molecular epidemiology of *P. vivax* in PNG, 574 isolates collected from four catchment sites in East Sepik ($N = 1$) and Madang ($N = 3$) Provinces were genotyped using the markers MS16 and *msp1F3*. Genetic diversity and prevalence of *P. vivax* was determined for all sites. Despite a *P. vivax* infection prevalence in the East Sepik (15%) catchments less than one-half the prevalence of the Madang catchments (27–35%), genetic diversity was similarly high in all populations ($H_e = 0.77$ – 0.98). High genetic diversity, despite a marked difference in infection prevalence, suggests a large reservoir of diversity in *P. vivax* populations of PNG. Significant reductions in transmission intensity may, therefore, be required to reduce the diversity of parasite populations in highly endemic countries such as PNG.

INTRODUCTION

With malaria elimination now firmly back on the global agenda, *Plasmodium vivax* is emerging as a substantial obstacle to progress.¹ Much remains to be understood regarding the biology, pathogenesis, and epidemiology of this parasite, which has long been neglected as a research priority, largely due to misclassification of *P. vivax* infections as benign.^{2,3} It is now thought that the global public health risk posed by *P. vivax* may be far greater than first anticipated as a result of its complex lifecycle, its broader global distribution than *P. falciparum*, and the potentially severe or fatal clinical disease associated with infection.⁴

Mapping the local and global population structure of *P. vivax* is essential both before and after the rollout of intervention measures and when establishing goals for elimination,^{5,6} as parasite populations can differ widely between locations due to vector prevalence, host genetics, and a variety of environmental factors.^{7–10} Population genetic surveys to determine the diversity of *P. vivax* parasites within a given human population can reveal insights into the potential resilience of the parasite population to interventions.

Additionally, as individuals infected with *P. vivax* are often infected with multiple clones, it is important to identify genetically distinct clones both between infections and within the same infection. Either one or two highly polymorphic markers or a larger number of less polymorphic genome-wide markers are generally used to genotype samples and identify distinct parasite clones. The diversity of a given marker and the number of markers required to accurately genotype *P. vivax* infections will, however, differ between geographical locations due to variations in malaria epidemiology.^{11–13} As such, the suitability of genotyping markers for a specific geographic location or region should be assessed before use. A panel of nine markers for genotyping *P. vivax* isolates was previously evaluated using samples collected in Papua New Guinea (PNG).¹³ Three markers—MS16, pv3.27, and *msp1F3*—were identified as the most genetically diverse in the PNG parasite population investigated and therefore, best suited to identify individual

clones. In a subsequent study by Koepfli and others,¹⁴ it was reported that using the unlinked MS16 and *msp1F3* markers combined was sufficient to identify genetically distinct clones from the highly endemic Maprik region in East Sepik Province of PNG, and also that the genetic diversity of *P. vivax* within this region is very high.¹⁴

The burden of *P. vivax* infection in PNG is the highest of any country in the world.¹⁵ More than 90% of the PNG population is at high risk of malaria,¹⁶ with the highest incidence of infection among children < 5 years.¹⁷ Despite 9 of 10 principle malaria-endemic countries in the Western Pacific region reporting downward trends in malaria in 2011, the number of malaria cases reported in PNG remained relatively stable.¹⁶ Furthermore, 36% of 262,000 malaria cases reported in the Western Pacific region in 2010 occurred in PNG, the highest burden of any single nation within the region.¹⁶ In PNG, transmission of the four major human malaria parasites (*P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale*) occurs year round.^{15,17,18} The intensity of malaria transmission ranges from epidemic outbreaks and low transmission in the highlands to holoendemic in the lowlands and coastal regions, which is similar in intensity to sub-Saharan Africa.¹⁵ Because of the enormous burden of *P. vivax* in PNG coupled with the inherent difficulties associated with *P. vivax* elimination, including the absence of a continuous *in vitro* culture system to investigate *P. vivax* biology and the formation of long-lived liver hypnozoites confounding attempts to interrupt transmission, progress to elimination is likely to be slow. The results obtained from studies performed to investigate *P. vivax* population genetic diversity and structure in distinct regions within PNG, where transmission intensity varies, will be useful in both a local (enabling the impact of subsequent interventions to be measured) and a global context (contributing to knowledge of *P. vivax* population complexity).

The aim of this study was to investigate the genetic diversity of *P. vivax* in the East Sepik and Madang Provinces of PNG and to compare this information with infection prevalence in the different areas. Archived *P. vivax* isolates collected in cross-sectional surveys of four distinct catchments were genotyped using the highly polymorphic markers MS16 and *msp1F3*. The genetic diversity found in each population was then measured based on these genotypes, and the results compared with the infection prevalence. The results have

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important implications for the control of *P. vivax* malaria in highly endemic regions.

MATERIALS AND METHODS

Study sites and *P. vivax* isolates. Madang and East Sepik Provinces are subject to intense perennial malaria transmission and have long been the focus of malaria research and control efforts.¹⁹ The catchment areas in these two provinces are separated by approximately 350 km of mountainous and swampy terrain, and overland travel between the two locations is not possible.²⁰ A map of PNG showing the location of the study sites can be found in the work by Schultz and others.⁸ To capture the diversity of the circulating parasite population, finger prick or venous blood was collected from asymptomatic volunteers of all ages in cross-sectional malaria surveys conducted as part of an Intermittent Preventative Treatment in Infants Trial.⁸ In East Sepik Province, 1,077 samples were collected in the relatively dry months between August and September of 2005 from individuals residing in the Wosera catchment area that encompasses seven villages spaced 2–10 km apart (Gwinyingi, Patigo, Kausaugu, Nindigo, Kitikum, Wisogum, and Tatemba). In Madang Province, 1,282 samples were collected in the rainy season of March of 2006 from individuals residing in three distinct catchment areas each encompassing one to five villages within 5–20 km of each other. These catchments include Mugil, comprising the villages of Dimer, Karkum, Matukar, and Bunu; Malala, including Amiten, Susure, Malala, Suraten, and Wakorma; and the area surrounding Utu health center. For the purposes of this study, samples collected from individual villages within specific catchment areas of East Sepik and Madang Provinces were combined to form geographically distinct populations for analysis, including two provincial populations (East Sepik and Madang) and four catchment populations (Wosera, Mugil, Malala, and Utu). Informed consent was obtained from all adult participants and parents or legal guardians of minors. Ethical approval to conduct this study was granted by the PNG Institute of Medical Research Institutional Review Board (No. 11-05), the Medical Research Advisory Committee of PNG (No. 11-06), the Alfred Hospital Research and Ethics Unit (No. 420-10), and the Walter and Eliza Hall Institute Human Research Ethics Committee (No. 11-12).

Genomic DNA was extracted from whole-blood samples using the 96-well QiaAmp DNA Extraction Kit (Qiagen, Chadstone, Victoria, Australia). Light microscopy (LM) and ligase detection reaction–fluorescent microsphere assay (LDR-FMA) were performed to identify samples infected with *P. vivax* as described.²¹ The proportion of samples that was LM- or LDR-FMA-positive for *P. vivax* infection was used to determine the infection prevalence for both provinces and each catchment. To increase the available template volume for analysis, whole-genome amplification (WGA) was performed using the Illustra GenomiPhi V2 DNA Amplification Kit as per the manufacturer's instructions (GE Healthcare, Rydalmere, New South Wales, Australia).

Genotyping. MS16 and *msp1F3* were selected to genotype *P. vivax* isolates in the present study, as it has been shown previously that diversity of the two markers is sufficiently high to enable high-resolution differentiation between genetically distinct strains.^{13,14} The MS16 marker is highly diverse and lacking in dominant alleles. Less diverse than MS16, the cod-

FIGURE 1. Genetic diversity of *P. vivax* populations on the north coast of PNG. Each wedge of the pie charts represents a single marker allele; the size of each wedge is proportional to the frequency of each allele within the specified population. The number of *P. vivax* isolates (n) from which markers were successfully amplified from each population is indicated.

ing sequence of the *msp1F3* marker is more complex than a microsatellite; therefore, artifacts introduced by slippage of the polymerase chain reaction (PCR) polymerase are less likely to occur.¹⁴

Genotyping was performed as previously described,¹⁴ with the following alterations: a multiplex first-round PCR was performed using the HotMaster Taq DNA Polymerase Kit (5 Prime, Murarrie, Queensland, Australia) and the published MS16 and *msp1F3* primer sequences of Koepfli and others.¹³ The primary PCR mastermix, in a final volume of 20 μ L, consisted of 1 μ L WGA template DNA, 2 μ L 10 $\times$ HotMaster Taq buffer with Mg^{2+} , 0.25 μ M each primer (Geneworks, Hindmarsh, South Australia, Australia), 0.2 mM deoxynucleotide triphosphates (dNTPs) (Roche, Castle Hill, New South Wales, Australia), and 1 U HotMaster Taq DNA polymerase (5 Prime). Thermocycling was performed under the following conditions: denaturation at 94°C for 2 minutes; 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, and primer extension at 65°C for 1 minute; final extension at 65°C for 5 minutes.

Nested PCR reactions were then performed on the primary product template using the HotMaster Taq DNA Polymerase

Kit (5 Prime) and either the MS16 or *mspIF3* nested PCR primer sequences of Koepfli and others.¹⁴ Nested PCR forward primers were labeled at the 5' end with a fluorescent dye, and nested PCR reverse primers were modified by the addition of a proprietary 7-bp tail (Applied Biosystems, Mulgrave, Victoria, Australia) to the 5' end as described.¹³ The nested PCR mastermix for both markers, in a final volume of 20 µL, consisted of 1 µL primary PCR template DNA, 2 µL 10× HotMaster Taq buffer with Mg²⁺, 0.25 µM forward and reverse primers (Applied Biosystems), 0.2 mM dNTPs (Roche), and 1 U HotMaster Taq DNA polymerase (5 Prime). Thermocycling was performed under the following conditions: denaturation at 94°C for 2 minutes; 25 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and primer extension at 65°C for 1 minute; final extension at 65°C for 5 minutes. Successfully amplified PCR products were then sent to a contract sequencing facility for fragment analysis using an ABI 3730XL automatic DNA analyzer and the Applied Biosystems 500LIZ size standard. The size standard 500LIZ was included in each sample lane.

Data analysis. GeneScan chromatograms were analyzed using Peak Scanner, version 1.0 (Applied Biosystems). A cutoff of 1,000 relative fluorescence units (RFUs) was used to distinguish true peaks from background signal. As a result of run-to-run variation, the fluorescence intensity of some sample plates was reduced, and in this instance, the cutoff was lowered to 300 RFUs.¹³ All major peaks (i.e., those peaks within the size range with the highest RFUs) were scored. A stringent peak calling strategy was used: secondary and/or minor peaks were only scored if they reached at least 40% of the major peak for *mspIF3* and 70% of the major peak for MS16. All scored peaks were visually inspected to confirm results.

Alleles were grouped manually according to size into bins of 3 bp, which is the size of the repeat unit for both MS16 and *mspIF3*.^{13,14} Isolates with single alleles for both markers were classified as single infections, whereas those isolates with multiple alleles for at least one marker were classified according to the highest number of alleles detected for either marker.

Diversity analysis was performed on the genotyping data using FSTAT software, version 2.9.3²² and Arlequin, version 3.5.1.2.²³ The number of alleles (*A*), the number of haplotypes (*h*), allele frequency and allelic richness (*R_s* is the expected number of alleles normalized on the basis of the smallest sample size)²⁴ were calculated for each catchment area, province and for PNG total. Expected heterozygosity (*H_e*), the probability of two clones randomly sampled from a given population carrying a different allele, was calculated using the formula

$$H_e = \frac{n}{n-1} \left(1 - \sum_{i=1}^n p_i^2 \right)$$

where *n* is the number of alleles identified in a given population and *p* is the frequency of allele *i*.

RESULTS

Prevalence of *P. vivax* on the north coast of PNG. A total of 2,359 blood samples was collected from asymptomatic par-

TABLE 1

Prevalence of *P. vivax* infections in the Madang and East Sepik regions of PNG

Province and catchment	<i>n</i>	<i>P. vivax</i> infection	
		LM-positive (%)	LDR-FMA-positive (%)
East Sepik	1,077	194 (18)	165 (15)
Wosera	1,077	194 (18)	165 (15)
Madang	1,282	211 (16)	409 (32)
Malala	379	70 (18)	131 (35)
Mugil	503	74 (14.7)	167 (33)
Utu	397	64 (16)	109 (27)
PNG total*	2,359	405 (17)	574 (24)

n, number of *P. vivax*-positive samples.

* The total number of positive samples for PNG and Madang include three samples from which the Madang catchment of origin was unknown.

ticipants from the East Sepik and Madang regions of PNG. Infection with *P. vivax* was determined using both LM and LDR-FMA. Of 2,359 samples, 405 (17%) samples were identified as *P. vivax*-positive using LM, whereas 574 (24%) samples were identified using the more sensitive molecular approach of LDR-FMA (Table 1). Of 574 *P. vivax* LDR-FMA-positive samples, 409 (71.3%) samples were from Madang (Table 1), and 165 (28.7%) samples were from East Sepik. Based on these data, Madang Province had an overall infection prevalence of 32% (catchments = 27–35%), which was more than double the infection prevalence of East Sepik Province (15%) (Table 1). LM data were not available for 81 samples: 12 samples from Madang and 69 samples from East Sepik.

Samples were collected from participants of all ages at both study sites. Ages ranged between < 1 month and 72 years (median = 14 years) among participants from Madang and between < 1 month and 90 years (median = 14 years) among participants from East Sepik. Demographic data were available for 442 of 574 *P. vivax* LDR-FMA-positive samples; 49% (*N* = 220) of *P. vivax*-positive samples were collected from children ages < 10 years, with 19.8% of samples collected from children ages < 5 years. Information regarding the gender of participants from East Sepik was not available.

Genotyping of *P. vivax* isolates. WGA, a method that has been used in similar molecular epidemiological studies,^{25–28} was performed prior to genotyping to increase the amount of *P. vivax* DNA for analysis. The multiple displacement amplification (MDA) method used in this study has been shown to result in a higher yield of non-artifact DNA template and reduced amplification bias compared with PCR-based WGA methods.²⁹ To investigate whether artifacts were introduced as a result of WGA, sequences of *mspIF3* and MS16 alleles amplified from undiluted genomic DNA and subjected to WGA were compared. Consistent with previous reports,^{26,27} results were concordant between the WGA and unamplified DNA (data not shown).

Of 574 *P. vivax*-positive samples, there was sufficient DNA to enable WGA and subsequent genotyping of 521 samples, of which 310 samples were PCR-positive for at least one of the genotyping markers. For 53 (17%) samples, MS16 only was successfully genotyped; for 63 (20.3%) samples, *mspIF3* only was successfully genotyped, and for 194 samples, both MS16 and *mspIF3* were successfully genotyped (Table 2).

Genetic diversity of *P. vivax* populations on the north coast of PNG. Among 310 *P. vivax* isolates, at least 487 individual clones were identified, and the alleles found amongst these clones were used to calculate diversity. The total number of

TABLE 2

Genetic diversity of *P. vivax* infections in the Madang and East Sepik regions of PNG

Markers in the province and catchment	<i>n</i>	<i>c</i>	<i>A</i>	<i>h</i>	<i>R_s</i>	<i>H_e</i>
MS16						
East Sepik	94	138	55	n.a.	55	0.98
Wosera	94	138	55	n.a.	47	0.98
Madang	152	237	69	n.a.	64	0.98
Malala	56	92	46	n.a.	43	0.97
Mugil	53	61	34	n.a.	34	0.96
Utu	43	84	43	n.a.	42	0.98
PNG total	246	375	82	n.a.	53	0.98
<i>mspIF3</i>						
East Sepik	87	103	17	n.a.	17	0.77
Wosera	87	103	17	n.a.	15	0.77
Madang	171	251	29	n.a.	24	0.86
Malala	54	92	16	n.a.	15	0.83
Mugil	72	94	15	n.a.	14	0.85
Utu	45	65	23	n.a.	23	0.91
PNG total	257	354	33	n.a.	20	0.85
Both						
East Sepik	75	124	30	69	n.a.	0.88
Wosera	75	124	30	69	n.a.	0.88
Madang	119	218	38	122	n.a.	0.92
Malala	40	77	20	40	n.a.	0.89
Mugil	44	70	20	51	n.a.	0.91
Utu	35	71	20	37	n.a.	0.91
PNG total	194	342	46	177	n.a.	0.99

A = number of alleles; *c* = number of clones counted; *h* = number of haplotypes; *H_e* = expected heterozygosity; *n* = number of *P. vivax* isolates; n.a. = not applicable; *R_s* = allelic richness.

alleles identified (*A*) was 82 for MS16 and 33 for *mspIF3* (Figure 1, Table 2, and Supplemental Table 1). The most common *mspIF3* allele was 265 bp in size and detected at a frequency of 30.8%. For MS16, the 377-bp allele was detected at the highest frequency of only 4.2% (Supplemental Table 1). Other parameters of diversity, including the expected heterozygosity (*H_e*) and allelic richness (*R_s*), were found to be high in all four catchments for each of the markers, although the range of values varied depending on the marker used, with *mspIF3* showing lower and more variable values of *R_s* and *H_e* because of its lower overall diversity (Table 2).

Very high levels of diversity were also observed after analysis of combined MS16–*mspIF3* haplotypes established from isolates considered to be single infections plus composite haplotypes from isolates with no more than two alleles at only one locus (*N* = 177) (Table 2). Despite a considerable difference in the *P. vivax* infection prevalence in Madang (32%) and East Sepik (15%) Provinces (Table 1), the genetic diversity was very high in both regions (*H_e* = 0.92 and *H_e* = 0.88, respectively). Similarly, in all four catchments investigated, the genetic diversity was very high (*H_e* = 0.88–0.91) (Table 2).

Although the use of two markers provides greater resolution to identify distinct clones, the genetic diversity measures of *A*, *R_s*, and *H_e* were not well-resolved among populations, which is also the case when extremely diverse markers, such as MS16, are used. Therefore, the analysis of *mspIF3* provided the greatest resolution of differences among the populations. Based only on *mspIF3*, *R_s* and *H_e* values were highest for Utu, which is interesting given the lower prevalence compared with the other two Madang catchments. *R_s* and *H_e* values were lowest for Wosera, which is consistent with the lower prevalence in that catchment (Table 2). In summary, all populations showed high levels of genetic

diversity, despite considerable variation in *P. vivax* infection prevalence.

DISCUSSION

Molecular epidemiology is extremely useful to investigate beyond the traditional measures of parasite prevalence afforded by LM, because it enables the identification of distinct clones. Consequently, genetic diversity can be measured as an indication of parasite population complexity and potential resilience of populations to interventions. Several molecular markers, including both polymorphic microsatellite and minisatellite repeats and single nucleotide polymorphisms, have been characterized and widely used for molecular epidemiological studies of *P. falciparum*.^{30,31} Although consensus markers are yet to be determined for *P. vivax*, MS16 and *mspIF3* were recently reported as being highly diverse after evaluation of a panel of potential *P. vivax* genotyping markers.¹³ MS16 has previously been used to assess the genetic diversity of *P. vivax* populations from Peru,³² Brazil,³³ Vietnam,¹² PNG,^{13,14} Sri Lanka, Ethiopia,²⁵ Myanmar,²⁵ and Korea.³⁴ The diversity of MS16 among *P. vivax* isolates from a range of global endemic areas is reported to be medium to high (*H_e* [range] = 0.5–0.9),^{12,14,25,32–34} with the exception of a single report of very low levels of diversity among isolates from Ethiopia (*H_e* = 0.19). This low level of diversity in Ethiopia was despite high diversity of other microsatellite loci, suggesting that MS16 might be contained within a selective sweep in the Ethiopian parasite population.²⁵ Fewer studies have been performed to investigate the genetic diversity of *mspIF3*^{13,35}; however, we and others have shown that use of the MS16 and *mspIF3* markers combined can enable high-resolution identification of genetically distinct clones.^{13,14} By using MS16 and *mspIF3* to genotype parasite isolates in the present study, we have shown that *P. vivax* populations in East Sepik and Madang Provinces on the north coast of PNG are extremely genetically diverse.

Samples analyzed in the present study were collected from asymptomatic individuals participating in a cross-sectional study. The typically low-parasite densities of asymptomatic *P. vivax* infections resulted in PCR being more sensitive for the detection of *P. vivax* than LM. A greater number of *P. vivax* samples from East Sepik were detected using LM compared with LDR-FMA (Table 1); however, this might be due to misclassification of some infections by the local microscopists. A third read of the microscopy slides would be required in the case of discrepant results observed from the first two reads (Barnadas C, personal communication). It was previously reported that the MS16 PCR assay was more sensitive than the PCR assay for *mspIF3*,¹⁴ but we observed the opposite; the proportion of samples found to be infected with *P. vivax* using only *mspIF3* was slightly higher (20.3%) than the proportion detected using only MS16 PCR (17%). However, consistent with the higher MS16 diversity reported in this and other studies,^{13,14} the number of clones detected using the MS16 PCR (*N* = 375) was slightly higher than the number of clones for *mspIF3* (*N* = 354) (Table 2 and Supplemental Table 1). In addition to the reduced diversity of *mspIF3* relative to MS16, the limiting concentrations of template DNA resulting in stochastic amplification of clones may also contribute to the discordant number of clones detected using the two markers.^{14,36} Despite performing WGA to increase sample volume, the

amount of *P. vivax* DNA in many samples remained at or below the threshold of MS16 and *msp1F3* PCR detection.

Very high genetic diversity of a *P. vivax* population from the Maprik region of East Sepik Province was recently reported.¹⁴ This region is approximately 15–20 km northwest of the Wosera catchment, one of four sites from which samples were collected for use in the present study. In Maprik, the diversity of both markers was very high, with MS16 diversity ($H_e = 0.97$) higher than the diversity of *msp1F3* ($H_e = 0.88$).¹⁴ No dominant MS16 alleles were detected, whereas detection of three dominant *msp1F3* alleles (238, 265, and 276 bp in size) at frequencies > 10% was reported.¹⁴ We report here that 238- and 265-bp *msp1F3* alleles were also detected at a frequency > 10%; however, the 259-bp allele was detected at a higher frequency (11.76%) than the 276-bp allele (8.68%) (Supplemental Table 1). In both this study and the study by Koepfli and others,¹⁴ the 265-bp allele was the most frequently detected *msp1F3* allele: a frequency of 24% in Maprik¹⁴ and a frequency of 30.8% in the present study. These varying allele frequencies could be caused by slight changes over time. All MS16 alleles were detected at a frequency of less than 4.2% (Supplemental Table 1). Combined, the results of this study and the results of the study by Koepfli and others¹⁴ show that *P. vivax* populations on the north coast of PNG are extremely diverse.

In recent years, there has been a significant decline in the prevalence of *P. falciparum* infection in the Wosera area.³⁷ This decline is thought to be a result of ongoing malaria studies in the region, heightened awareness of malaria prevention measures, and increased access to treatment. Most importantly, however, the Wosera area was targeted for a community-wide distribution of long-lasting insecticide treatment nets by a local non-governmental agency. As a consequence, > 97% of participants surveyed from the Wosera catchment reported using bed nets. Although bed net usage also ranged from 71% (Mugil catchment) to 98% (Malala catchment) in the Madang areas, the bed nets used there were neither treated nor long lasting (Mueller I and others, unpublished data). In a large cross-sectional survey in East Sepik Province conducted 5 months before the present survey, high bed net coverage was associated with a reduction in prevalence of all four human *Plasmodium* spp.¹⁷ Hence, it is likely that increased insecticide-treated bed net usage has contributed to reduced *P. vivax* prevalence in East Sepik compared with the Madang villages. Although differences in transmission intensity due to seasonal variations cannot be discounted as samples from East Sepik Province were collected during the dry season and samples from Madang Province were collected in the rainy season, surveys conducted in the Wosera in 2001–2003 prior to the mass distribution of insecticide-treated bednets revealed very little difference in the prevalence of *P. falciparum* and *P. vivax* infections between the wet and dry seasons.³⁷ Furthermore, because infection with *P. vivax* can result in production of latent hypnozoites, *P. vivax* would be expected to be less sensitive to seasonal variations compared with *P. falciparum*. Indeed, sympatric populations of *P. falciparum* surveyed in the same study showed a wider range of infection prevalence among villages of the Wosera (range = 1.7–43.5%).³⁸ Additionally, the villages comprising the three Madang catchments are distributed over a broader geographical area (> 50 km) than those villages of the Wosera (< 10 km).⁸ Therefore, although likely due to higher coverage of insecticide-

treated bed nets, local differences in vector abundance and ecology may also have contributed to differences in relative *P. vivax* prevalence¹⁹ and warrant future investigation.

It is an important observation that, despite the much lower infection prevalence, genetic diversity of the East Sepik *P. vivax* population was similar to that of the Madang population. A similar observation was recently reported after analysis of *P. falciparum* infections from the Peruvian Amazon³⁹ and the same areas analyzed in this study.³⁸ For *P. vivax*, high genetic diversity may be maintained in the face of reduced transmission as a result of the latent hypnozoite stage of the lifecycle, which can remain in the liver for several months and even years.³³ Infection with different clones is thought to increase population genetic diversity through meiotic recombination in the mosquito midgut.^{12,33} Such genetic diversity is essential for parasite fitness and survival, and it is thought that this diversity must be reduced to limit parasite adaptation and population expansion.^{5,40} Thus, although the implementation of malaria prevention measures seem to have reduced *P. vivax* prevalence in East Sepik, an even greater reduction in prevalence may be required to decrease population genetic diversity for both *P. vivax* and *P. falciparum*.³⁸ The genetic complexity and unique biology of *P. vivax* infections, coupled with the enormous burden of both species, will present major challenges for malaria elimination in PNG.

Despite the successful implementation of malaria interventions in the East Sepik Province of PNG, a high level of *P. vivax* genetic diversity was observed, although the infection prevalence was less than one-half that recorded for Madang Province. A similar finding has been observed for sympatric *P. falciparum* populations.³⁸ High levels of genetic diversity, despite a relatively low infection prevalence in the Wosera, which was sampled during the dry season, show that *P. vivax* and *P. falciparum* parasite populations are well-equipped to adapt to a changing local environment and that a significant reduction in transmission intensity will be required to reduce population genetic diversity in highly endemic areas such as PNG. The results of the present study highlight the enormous challenges facing malaria elimination efforts in countries of high malaria endemicity such as PNG.

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