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Higher Complexity of Infection and Genetic Diversity of *Plasmodium vivax* than *Plasmodium falciparum* across all Malaria Transmission Zones of Papua New Guinea

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Abstract. *Plasmodium falciparum* and *Plasmodium vivax* have varying transmission dynamics that are informed by molecular epidemiology. This study aimed to determine the complexity of infection and genetic diversity of *P. vivax* and *P. falciparum* throughout Papua New Guinea (PNG) to evaluate transmission dynamics across the country. In 2008–2009, a nationwide malaria indicator survey collected 8,936 samples from all 16 endemic provinces of PNG. Of these, 892 positive *P. vivax* samples were genotyped at *PvMS16* and *PvmspF3*, and 758 positive *P. falciparum* samples were genotyped at *Pfmsp2*. The data were analyzed for multiplicity of infection (MOI) and genetic diversity. Overall, *P. vivax* had higher polyclonality (71%) and mean MOI (2.32) than *P. falciparum* (20%, 1.39). These measures were significantly associated with prevalence for *P. falciparum* but not for *P. vivax.* The genetic diversity of *P. vivax* (*PvMS16*: expected heterozygosity = 0.95, 0.85–0.98; *PvMsp1F3*: 0.78, 0.66–0.89) was higher and less variable than that of *P. falciparum* (*Pfmsp2*: 0.89, 0.65–0.97). Significant associations of MOI with allelic richness (rho = 0.69, P = 0.009) and expected heterozygosity (rho = 0.87, $P < 0.001$) were observed for *P. falciparum*. Conversely, genetic diversity was not correlated with polyclonality nor mean MOI for *P. vivax*. The results demonstrate higher complexity of infection and genetic diversity of *P. vivax* across the country. Although *P. falciparum* shows a strong association of these parameters with prevalence, a lack of association was observed for *P. vivax* and is consistent with higher potential for outcrossing of this species.

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by female anopheline mosquitoes.[1] Globally, an estimated 3.2 billion people are at risk of developing malaria. Despite increased global efforts to control and eventually eliminate malaria, it is still one of the major health problems causing an estimated 214 million new cases and 438,000 deaths in 2015 alone.[2] *Plasmodium falciparum* and *Plasmodium vivax* cause the majority of this malaria burden. Although *P. falciparum* is the more virulent of the two species, *P. vivax* has a wider geographic range and is more difficult to control.[3–5] In areas where *P. falciparum* and *P. vivax* are co-endemic, control efforts have had less impact on *P. vivax*, resulting in it becoming the more dominant infection in the community in some areas.[2] The main reason for this shift might lie in malaria control measures largely targeted at *P. falciparum*, and that *P. vivax* is more difficult to treat and diagnose because of its unique biology, in particular its ability to form dormant liver-stage infections known as hypnozoites.[3] *P. vivax* is, therefore, recognized as a major obstacle to the control and elimination of malaria in co-endemic regions.[6–9]

Coinfection or superinfection of hosts with multiple *Plasmodium* strains (also known as multiple infection) is common in endemic areas, which may result from the bite of mosquitoes infected with more than one clone or from multiple bites, respectively.[10,11] Multiple infection is a prerequisite for sexual recombination between different strains (outcrossing), which takes place within the mosquito midgut after ingestion of an infected blood meal. Interaction and competition of different strains for limited resources within a host during their life cycles may be important for survival.[12] Although poorly understood, this competition to survive might lead to increased virulence,[13] higher transmissibility,[14,15] and emergence of drug resistance.[16–19] High proportions of multiple infections and high genetic diversity of *P. vivax* even in low prevalence areas indicate that *P. vivax* populations are more stable under pressure and that more intensive and sustained interventions will be needed to control and eventually eliminate this parasite.[20–22] Measuring the complexity of infection and genetic diversity of parasite populations across different endemicities may be used as an indicator to evaluate the efficacy of control and elimination strategies.[23]

The burden of malaria in Papua New Guinea (PNG) is among the highest in the Asia Pacific region.[5,8,24] Four of the five malaria species known to infect humans, *P. falciparum*, *P. vivax*, *Plasmodium malariae*, and *Plasmodium ovale* s.l., are endemic in PNG. The intensity of malaria transmission is geographically variable even on small spatial scales with *P. falciparum* and *P. vivax* accounting for the majority of infections.[25–28] From 2004 to 2009, PNG conducted the first country-wide free distribution of long-lasting insecticide treated mosquito nets (LLINs) supported by the Global Fund to Fight AIDS, Tuberculosis and Malaria.[29] After the initial roll out of LLIN, a countrywide malaria indicator survey was undertaken to assess malaria prevalence in 49 villages from 16 provinces (of a total of 20), representing all malaria endemic areas of PNG. Additionally, 19 villages were included from six sentinel sites, which had not yet been covered by LLINs. Malaria prevalence, based on light microscopy, was 12% overall but varied widely between areas (0–49.7%) and *P. falciparum* was more prevalent than *P. vivax* in most areas. Lower prevalence in some regions

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was associated with LLIN use, strongly supporting the application of this strategy to control malaria in PNG.[30]

New tools are needed to monitor the success of malaria control programs. Molecular epidemiology can provide such a tool, by providing deeper insight into parasite transmission intensity than traditional prevalence surveys by measuring the complexity of infection and population genetic diversity accumulating as a result of co-transmission of multiple genetically distinct clones. Such studies have demonstrated that *P. vivax* is more genetically diverse than *P. falciparum*, with more complex infections,[31–37] but it is not well understood how these parameters relate to endemicity for *P. vivax*. Previous studies in PNG have focused on the highest transmission region on the north coast of PNG, namely the East Sepik and Madang Province.[38–42] Therefore, we aimed to gather genotyping data from all areas covered in the national malaria indicator survey mentioned earlier. Using this data, we have compared the molecular epidemiology of *P. vivax* and *P. falciparum* populations from all malaria-endemic areas of PNG representing a range of transmission intensities.

MATERIALS AND METHODS

Study sites and samples. A total of 8,936 samples were collected during a household-based national malaria indicator survey between October 2008 and August 2009 (Table 1). This survey included 49 villages from 16 provinces, which had already covered by free LLIN and 19 villages from six sentinel sites pre-LLIN distribution.[30] Further details of the survey methodology are published elsewhere.[29,30] We assigned the villages to 27 "geographic clusters" predicted to harbor distinct parasite populations on the basis of topography and predicted human movement (Figure 1, Supplemental Table 1). Genomic DNA was extracted from whole-blood samples using the QiaAmp DNA Extraction Kit (Qiagen, Chadstone, Victoria, Australia) or the Favorprep™ genomic DNA extraction kit (Favorgen, Ping-Tung, Taiwan). Light microscopy (LM) and ligase detection reaction fluorescent microsphere assay (LDR-FMA) were performed to identify samples infected with different *Plasmodium* species.[43]

Polymerase chain reaction and genotyping. *PvMS16* and *Pvmsp1F3* markers were used to genotype *P. vivax* positive samples (N = 1,339) since the use of these two highly diverse markers combined has previously demonstrated the ability to track clones, measure multiplicity of infection (MOI), and to provide insight into parasite genetic diversity within populations.[39,44] *Plasmodium vivax* samples were genotyped by multiplex first-round and secondary nested polymerase chain reaction (PCR) as per published protocols.[42,44] *Plasmodium falciparum* positive samples (N = 1,513) were genotyped using the highly polymorphic *Pfmsp2* marker as previously described.[45] A nested multiplex PCR approach was used to amplify 3D7 and/or FC27 family alleles of the gene encoding the highly polymorphic antigen, *Pfmsp2*, using family-specific fluorescent dye–labeled primers.

For all markers, PCR products were run on a 2% w/v agarose gel to check for bands within the correct size range and 10 μL of each positive PCR product was sent to a

Table 1
Summary of parasite isolates and genotyped samples

Cluster no.	Cluster name	*N*	*Pv*+	*PvMS16*	*Pvmsp1F3*	*Pf*+	*Pfmsp2*
1	West Sepik: Yapsie area	534	39	25	31	66	25
2	West Sepik: Aitape area	167	34	27	30	46	34
3	East Sepik: Angoram	254	41	34	39	16	10
4	Madang-Bogia	264	37	27	33	60	51
5	Madang: Ramu Valley	334	150	98	124	60	34
6	Morobe: Markham Valley	143	47	55	39	60	104
7	Morobe: Bulolo	76	37	20	26	37	6
8	Morobe: Mumeng area	203	54	39	48	75	21
9	Morobe: Huon Peninsula	797	118	98	118	127	47
	Overall Momase	**2,772**	**557**	**423**	**488**	**547**	**332**
10	Eastern Highlands	450	79	77	70	70	26
11	EHP: Obura-Wonenara	90	19	19	19	3	nd
12	WHP: Angalimp-South Waghi	143	20	nd	–	30	30
13	Western Highlands	324	33	38	40	43	10
	Overall Highlands	**1,007**	**151**	**134**	**129**	**146**	**66**
14	Gulf cluster no. 1	274	9	4	3	51	8
15	Gulf cluster no. 2	243	0	nd	–	9	nd
16	Oro (northern)	773	68	17	25	218	30
17	Central Coastal	613	56	75	95	49	4
18	Western: Balimo area	244	76	46	57	10	3
19	Western: Wipim area	368	39	27	37	36	4
20	Milne Bay	200	7	nd	–	4	nd
21	Milne Bay: Kiriwina	105	10	nd	–	13	7
	Overall Southern	**2,820**	**265**	**169**	**217**	**390**	**56**
22	Manus	303	97	64	73	60	34
23	WNB: Kimbe Bay	352	30	15	24	80	63
24	WNB: South coast	370	67	48	49	102	88
25	ENB: Gazelle Peninsula	443	80	49	52	75	33
26	New Ireland	439	62	73	83	81	79
27	Bougainville	430	30	22	28	32	7
	Overall Island	**2,337**	**366**	**271**	**309**	**430**	**304**
	Total	**1,339**	**997**	**1,143**	**1,513**	**758**	**8,936**

EHP = Eastern Highlands Province; ENB = East New Britain; LDR-FMA = ligase detection reaction fluorescent microsphere assay; N = Number of samples; nd = not done; *Pv*+ = number of samples *Plasmodium vivax* positive by LDR-FMA; *Pf*+ = number of samples *Plasmodium falciparum* positive by LDR-FMA; WHP = Western Highlands Province; WNB = West New Britain. Dashes indicate that no samples were successfully genotyped. Bold values are to highlight them as aggregated populations.

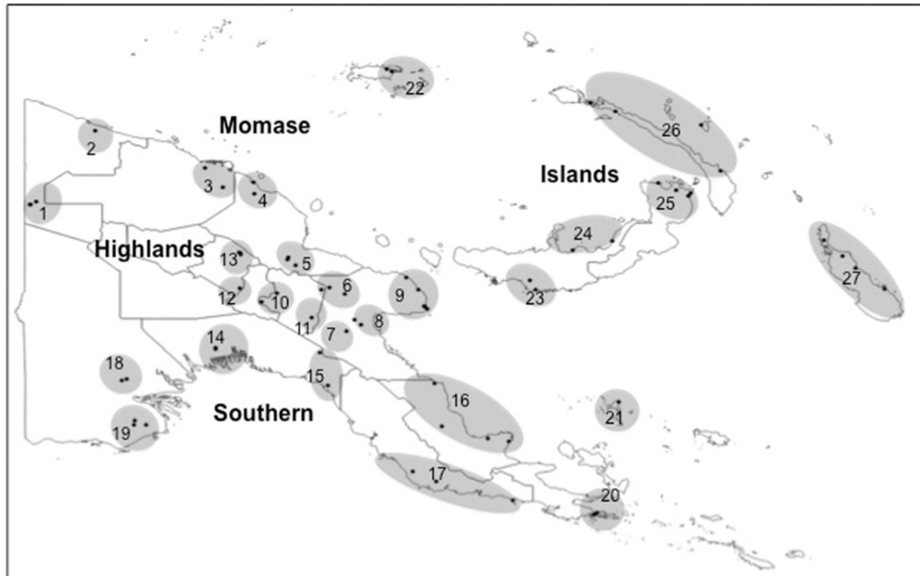

FIGURE 1. Map of the study area. Dots indicate sampled villages, shaded areas indicate the geographic clusters predicted to harbor distinct parasite populations. Broad geographic regions are indicated: Momase (clusters 1–9), Highlands (clusters 10–13), Southern (clusters 14–21), and Islands (clusters 22–27). See Supplemental Table 1 for names of clusters and villages.

commercial sequencing facility for fragment analysis (GeneScan, Applied Biosystems, Foster City, CA,). The resulting chromatograms were analyzed using GeneMapper® Software Version 4.0 (Applied Biosystems) calibrating against the internal size standard GSLIZ500. A cutoff of 1,000 relative fluorescence units (RFUs) were used to distinguish true peaks from background signal and to adjust run-to-run variation, the fluorescence intensity of some sample plates was reduced, and in this instance, the cutoff was lowered to 300 RFU. All major peaks (i.e., those within the size range with the highest RFUs) and any additional alleles with minimum 33% height of the predominant allele were scored. All traces were inspected manually to confirm call quality.

Data analysis. We determined the prevalence of infection by dividing the total number of positive infections (*Pv*+ or *Pf*+) by the number of samples screened (*N*). Age-adjusted *P. vivax* and *P. falciparum* prevalence data for 2- to 10-year-olds ($PvPR_{2–10}$ and $PfPR_{2–10}$) were used to determine the association between parasite prevalence with other genetic diversity parameters.[46] Capillary electrophoresis allowed high-resolution detection of the number of parasite clones, i.e., MOI within each isolate (sample) based on the number of alleles detected for the respective markers. As two markers were used for *P. vivax*, MOI was based on the maximum number of alleles detected for either marker. The number of clones (*c*) for each population was determined by summing the total number of clones per isolate. Mean MOI for each population was calculated by dividing the total number of clones detected by the number of samples positive for the relevant marker/s (*n*). Another measure of the complexity of infection, polyclonality, was determined by calculating the proportion of samples carrying multiple infections (MOI > 1) in each population. 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 maximum number of alleles counted for either marker.

Diversity analyses were performed on the genotyping data using R package "Hierfstat," (http://www.unil.ch/popgen/softwares/hierfstat.htm) and FSTAT software version 2.9.3.[47] The number of alleles (*A*), allele frequency, expected heterozygosity (H_e), and allelic richness (R_s) were calculated for each parasite population in different geographic areas in PNG. The expected heterozygosity, represents the probability of being infected by two parasites of the same species with different alleles at a given locus and was calculated using the following formula: $H_e = [n/(n-1)]\,[(1 - \Sigma p_i^2)]$, where *n* is the number of isolates sampled and p_i is the allele frequency (*p*) at the *i*th locus. Allelic richness considers the average number of alleles normalized for the smallest sample size among the sampled populations based on the rarefaction method of Hurlbert and others.[48] Spearman's rho was used to measure associations between prevalence, complexity of infection, and diversity parameters. χ^2 tests or one-way analysis of variance were used to measure differences among two groups or more than two groups, respectively. A *P* value of ≤ 0.05 was considered statistically significant. Statistical analyses were done using GraphPad Prism Software version 6.0.

RESULTS

Prevalence and complexity of infection of *P. vivax* and *P. falciparum* throughout PNG. A total of 1,339 *P. vivax*- and 1,513 *P. falciparum*-infected samples were identified by LDR-FMA (Table 1). Of these, 892 *P. vivax* samples were successfully genotyped with *PvMS16* and *Pvmsp1F3* markers, and a total of 3,059 clones were identified (Table 1, Supplemental Table 2). Overall, there was no significant difference in the number of clones counted, mean MOI, and polyclonality between *PvMS16* and *Pvmsp1F3* analyzed individually or in combination (Supplemental Figure 1, Supplemental Table 2). For *P. falciparum*, among 758 samples successfully genotyped with *Pfmsp2*, relatively low numbers of clones

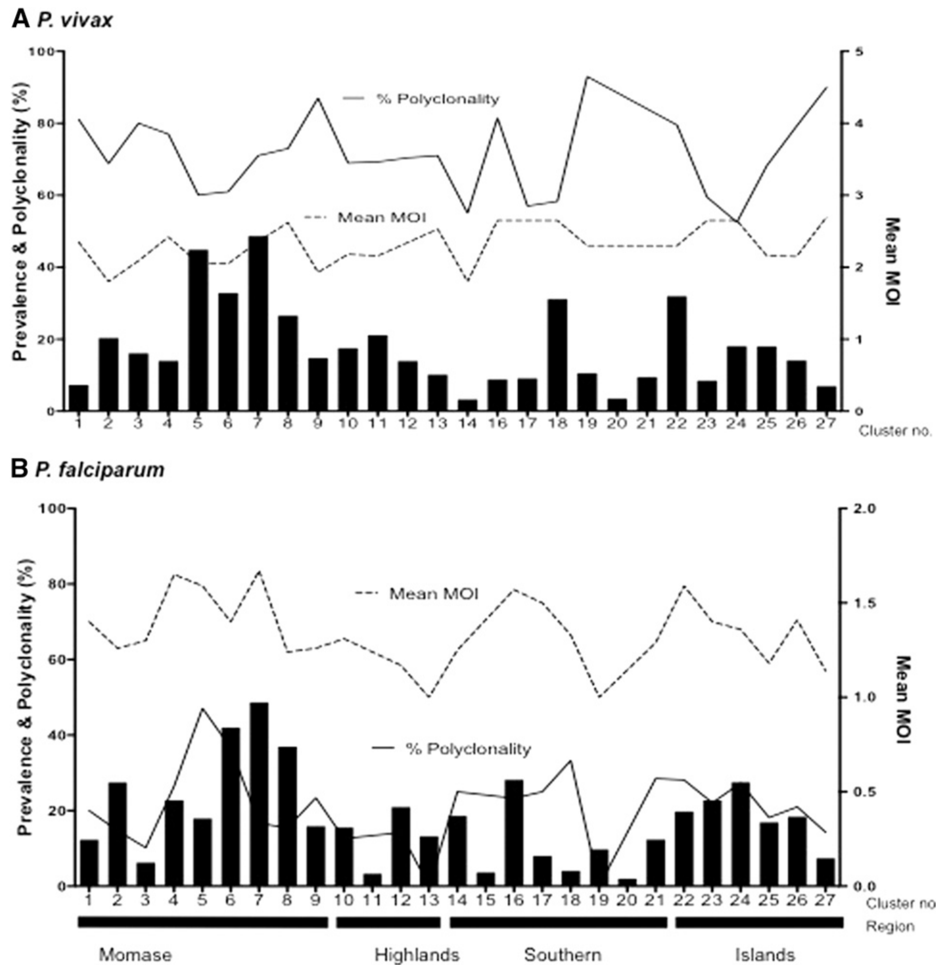

FIGURE 2. Complexity of infection in different geographic clusters and regions of Papua New Guinea. Proportion of polyclonal infections (solid line) and mean multiplicity of infection (MOI) (dashed line) relative to prevalence (all ages) in different geographic clusters and regions for (**A**) *Plasmodium vivax* and (**B**) *Plasmodium falciparum.*

(c = 1,049) were identified (Table 1, Supplemental Table 3). Of these, 79.8% of clones had 3D7-type *Pfmsp2* alleles and 21.2% had FC27-type *Pfmsp2* alleles.

Despite significant variability of *P. vivax* prevalence among geographic areas,[30] polyclonality (mean = 70.8%, range = 52.5–93.0%) and MOI (mean = 2.32, range = 1.8–2.7) based on the two markers combined were high in all geographic areas (Figure 2A, Supplemental Table 2). For *P. falciparum*, lower and more variable polyclonality (mean = 21.5%, range = 0–47.1%) and mean MOI (mean = 1.4, range = 1–1.6) were

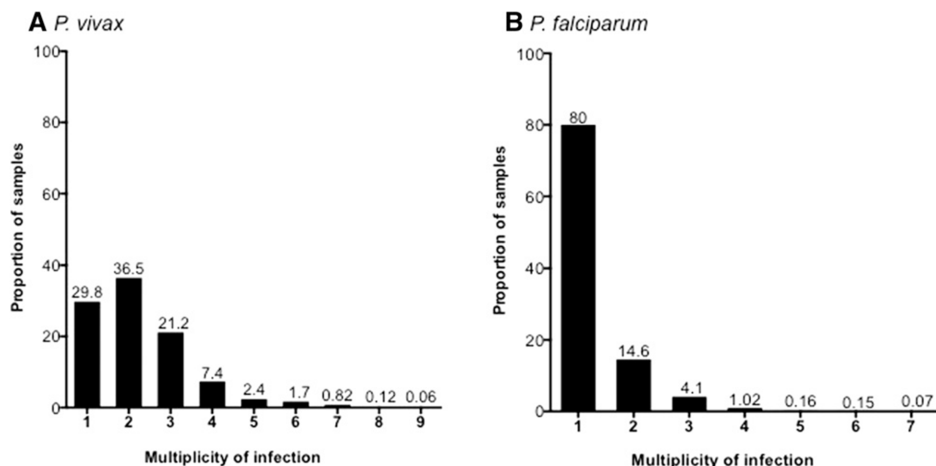

FIGURE 3. Frequency distribution of multiplicity of infection in Papua New Guinea. Frequency of multiplicity of infection (MOI) values among all samples for (**A**) *Plasmodium vivax* and (**B**) *Plasmodium falciparum.* Numbers above the columns indicate proportions.

observed (Figure 2B, Supplemental Table 3). Aggregating the geographic clusters into the four major geographical regions of PNG (Momase, Highlands, Southern, and Islands), *P. vivax* had very high polyclonality in all regions (68.9–73.2%) (Figure 2A, Supplemental Table 2), whereas for *P. falciparum*, the Momase region, encompassing the entire north coast where prevalence is high, had the highest polyclonality (23.4%) and mean MOI (1.45) while the Highlands region, where prevalence was lower, had very low polyclonality (19.6%) and the lowest MOI (1.2) (Figure 2B, Supplemental Table 3).

The frequency distribution of multiple infections was also different between the two species. For *P. vivax*, only 29.3% samples had single infections, most of the samples had two clones (36.5%) and a large proportion of infections had three clones (21.2%), four clones (7.4%), and greater than four clones (5.1%) (Figure 3A). Whereas, the majority of *P. falciparum* samples had single infections (80%), with most of the remaining samples having only two clones (14.6%) and only a small proportion had three clones (4%) or greater (1.4%) (Figure 3B). For *P. vivax*, a maximum of nine clones were identified in a sample, whereas for *P. falciparum*, there was a maximum of seven clones.

Genetic diversity of *P. vivax* and *P. falciparum* populations throughout PNG. For *P. vivax*, a total of 111 *PvMS16* and 60 *Pvmsp1F3* alleles were identified with varying alleles and allele frequencies observed in the parasite populations of different geographic clusters. The most frequent allele identified for *Pvmsp1F3* marker was the 268 bp allele (34%) followed by 241 (25%), 256 (12%), and 262 bp (9%) alleles (Supplemental Figure 2A). In contrast, the *PvMS16* marker showed a large number of low frequency (< 10%) alleles. The most common allele identified was allele with 269 bp (8%) followed by 377 (6%), 242 (5%), and 263 bp (3%) (Supplemental Figure 2B). For *P. falciparum*, a total of 105 *Pfmsp2* alleles were identified with the 348 bp allele being the most predominant (11%) followed by 252 (9%), 175 (8%), and 486 bp (6%) alleles. A variable number of alleles unique to different populations were observed (Supplemental Figure 2C).

Plasmodium vivax had high genetic diversity (*PvMS16*: mean R_s = 22, range = 15–25, based on a minimum sample size of 15, mean H_e = 0.95, range = 0.85–0.98; *msp1F3*: R_s = 9, 5–13 based on a minimum sample size of 24, H_e = 0.78, 0.66–0.90; Figure 4A, Supplemental Table 4). The genetic

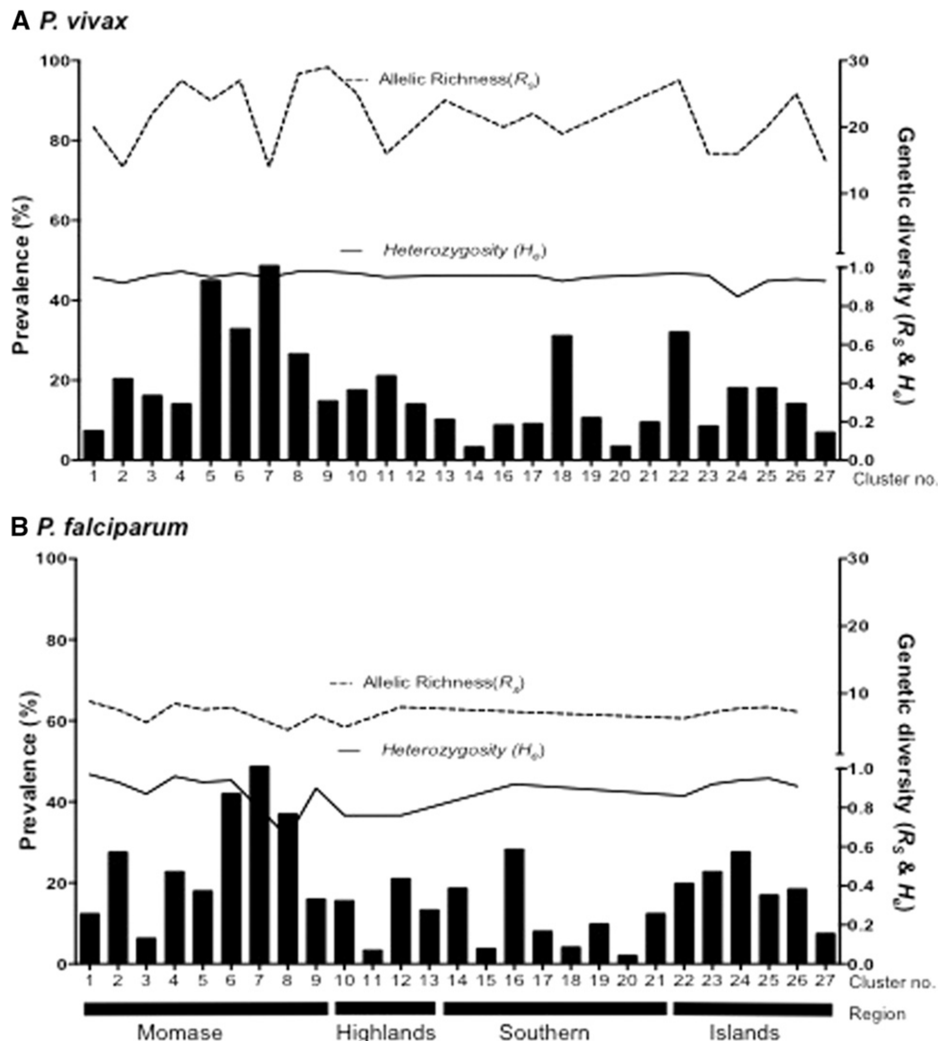

FIGURE 4. Genetic diversity of *Plasmodium vivax* and *Plasmodium falciparum* in different geographic clusters and regions of Papua New Guinea. Genetic diversity as measured by heterozygosity (solid line) and allelic richness (dashed line) relative to prevalence (all ages) in different geographic clusters and regions for (**A**) *Plasmodium vivax* (*PvMS16*) and (**B**) *Plasmodium falciparum* (*Pfmsp2*).

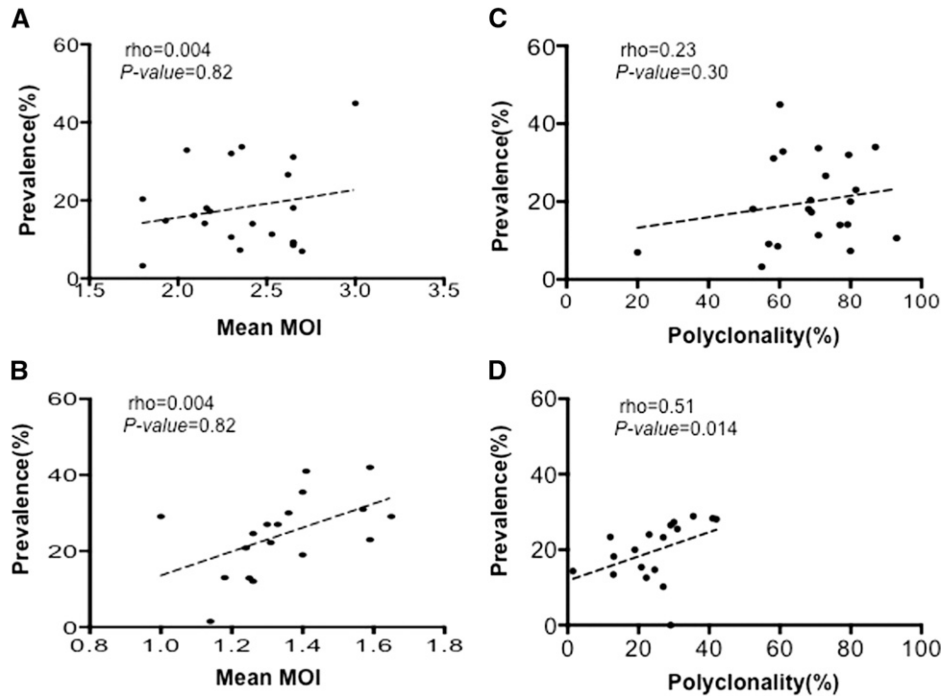

FIGURE 5. Association between parasite prevalence and infection complexity in different geographic clusters of Papua New Guinea. Association between age-corrected prevalence and mean multiplicity of infection (MOI) for (**A**) *Plasmodium vivax* and (**B**) *Plasmodium falciparum*. Association between age-corrected prevalence and polyclonality for (**C**) *P. vivax* and (**D**) *P. falciparum*. The dashed lines indicate the best fit for Spearman's rho and *P* values indicated on each plot.

diversity of *Pfmsp2* in *P. falciparum* populations across all geographic areas was also high (R_s = 7, 4.6–8.8 based on a minimum sample size of 10, H_e = 0.89, 0.65–0.97), except for Morobe, Mumeng (cluster 8). This suggests that despite high prevalence, this population may be genetically isolated relative to the others (Figure 4B, Supplemental Table 5). Higher diversity of *P. vivax* was observed for both markers based on R_s, and for *PvMS16* using H_e. As H_e is an allele frequency–based

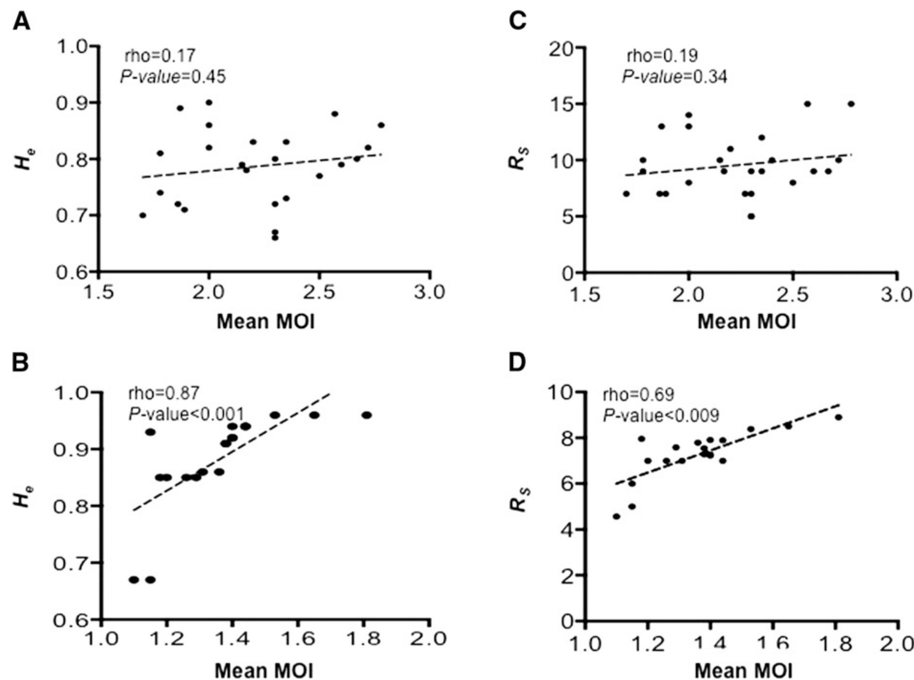

FIGURE 6. Association between infection complexity and genetic diversity in different geographic clusters of Papua New Guinea**.** Association between mean multiplicity of infection (MOI) and heterozygosity (H_e) for (**A**) *Plasmodium vivax* (**B**) *Plasmodium falciparum*. Association between mean MOI and allelic Richness (R_s) for (**C**) *P. vivax* and (**D**) *P. falciparum*. The dashed lines indicate the best fit for mean MOI and genetic diversity with Spearman's rho and *P* values indicated on each plot.

measure, this likely reflects the different allele frequency distributions of the two *P. vivax* markers.

Relationship between prevalence, complexity of infection, and parasite genetic diversity. *Plasmodium vivax* had high polyclonality and mean MOI in all parasite populations despite different parasite prevalence, consistent with high complexity of *P. vivax* infections within the host regardless of endemicity and prevalence in different areas. Spearman's rank correlation analysis showed neither mean MOI nor polyclonality was significantly associated with age-corrected *P. vivax* prevalence (Figure 5A and C). However, for *P. falciparum*, polyclonality and mean MOI showed a statistically significant correlation with age-corrected prevalence ($P < 0.02$; Figure 5B and D).

Plasmodium vivax genetic diversity measures (H_e and R_s) were not significantly associated with age-corrected prevalence (rho = 0.016, $P = 0.94$) nor mean MOI (Figure 6A and C). *Plasmodium falciparum* genetic diversity also did not show any association with age-corrected prevalence (rho = 0.21, $P = 0.38$); however, both allelic richness and expected heterozygosity showed a highly significant positive correlation with mean MOI (Figure 6B and D).

DISCUSSION

The molecular epidemiology of the sympatric malaria parasite species *P. falciparum* and *P. vivax* throughout all endemic regions of PNG highlights their contrasting biology and the potential of complexity of infection and genetic diversity as markers of transmission intensity, at least for *P. falciparum*. The greater complexity of infection of *P. vivax* across a wide range of endemicities illustrates its higher potential for outcrossing and subsequent generation of genetic diversity, which was high in all populations and higher than *P. falciparum* as described in other studies conducted on the north coast of PNG[39,42,49] and in Asia.[33,50–52] The complexity of infection was somewhat variable among different regions and geographic clusters for both species, and is comparable to that found in a wide range of higher transmission settings in other countries.[27,28,33,38,53–57] However, the vast majority of *P. vivax* infections were polyclonal and MOI was almost 2-fold higher than that of *P. falciparum*. Furthermore, although complexity of infection was associated with prevalence for *P. falciparum*, there was no association between these parameters for *P. vivax.* Genetic diversity was high throughout the country for both species and was associated with MOI for *P. falciparum* but not for *P. vivax*, which indicates that diversity at the *P. vivax* loci studied is close to saturation point across all malaria-endemic areas of PNG. The greater complexity of infection and higher diversity of *P. vivax* are likely the results of recurrent blood-stage infection due to relapse in this species, which account for 80% of all blood-stage infections in PNG.[58]

The complexity of infection has implications for testing drug efficacy, disease severity, and population diversity.[59,60] Therefore, accurate detection of parasite clones circulating within the host is crucial and depends on the assay sensitivity, the type and number of the molecular markers analyzed, and the criteria used to identify predominant and minor alleles.[55,61] The use of relaxed criteria to score minor peaks significantly increases artifacts that falsely increase multiclonal infections.[62] Because the markers used are highly diverse, we used more stringent criteria to minimize the introduction of artifacts (see Methods). The prevalence of polyclonal infections and mean MOI in the separate analyses of the *P. vivax* markers *PvMS16* and *Pvmsp1F3* was similar to the two markers in combination, demonstrating that either marker could have been used to describe this aspect of *P. vivax* epidemiology. The similar total number and frequency distribution of *PvMS16* and *Pfmsp2* alleles warrants the use of these markers alone. However, *Pvmsp1F3* was included because it, like *Pfmsp2*, is an antigen, with variation influenced by immune selection.[63,64] When the diversity of *Pfmsp2* and *Pvmsp1F3* was compared, *P. vivax* diversity based on the allelic richness within each parasite population was higher, despite fewer alleles overall in the *P. vivax* marker. However, the expected heterozygosity of *Pvmsp1F3* was lower than that of *Pfmsp2*. Other studies have shown that *Pvmsp1* has limited diversity compared with *msp1* in other *Plasmodium* species[65] and the lower overall diversity compared with *Pfmsp2* suggests its functional requirements may restrict diversity. Immune (balancing) selection[66] or recent reductions in population size due to control activities[64] may also influence the diversity measures differently. An additional reason for using two markers for *P. vivax* is that the detectability of clones may be lower than that of *P. falciparum* due to this species causing lower density infections.[67] Based on the markers used, the average number of clones per infection for *P. vivax* was almost double that of *P. falciparum*, and would be even higher if clones were missed for this reason. Therefore, the use of the two chosen markers on different chromosomes for *P. vivax* is valid and increases the accuracy of the MOI and diversity measurements described here.

Based on the combination of *PvMS16* and *Pvmsp1F3* markers, *P. vivax* had a very high proportion of polyclonal infections with 70% of all genotyped samples having more than one clone and a high mean MOI of 2.3. For *P. falciparum Pfmsp2*, however, only 20% of all genotyped samples had polyclonal infections and there was a much lower mean MOI of 1.39, despite the higher prevalence of *P. falciparum* than *P. vivax* in many areas.[30] The contrasting complexity of infection for the two species is comparable to previous reports from the PNG north coast[39,68] and demonstrates that this pattern is also observed across areas with widely varying endemicity including areas with low parasite prevalence.[30] This may be in part due to lower transmission areas having increasing proportions of imported infections, which reflects the MOI and diversity of the infection origin,[22,69,70] and this effect would be enhanced for *P. vivax* by the fact that relapse can occur 1–3 years after the primary infection.[71] The high complexity of infection for *P. vivax* regardless of parasite prevalence is also comparable to other studies where high infection complexity and diversity were found even with sustained low parasite prevalence such as in South America[33,72,73] and Sri Lanka.[74,75] Polyclonal infections are common in malaria-endemic areas of different countries for both species and can arise from a single mosquito bite carrying multiple clones or from inoculation by different mosquitoes carrying single clones. However, relapses of *P. vivax* infection due to the reactivation of heterologous hypnozoites from previous infections can directly contribute to increased MOI, which may sustain high diversity in low transmission areas for a longer period than *P. falciparum* after reduction in transmission.[3,22,61] This unique biology thus may explain the high complexity of *P. vivax* infection at a range of parasite prevalences in PNG.

Some of the results suggest that malaria control initiatives in PNG prior to the survey including LLIN and indoor residual spraying in some areas[29,30] have had a greater impact on *P. falciparum*. The highest mean MOI and polyclonality for *P. falciparum* was recorded in a parasite population from a sentinel site (Morobe: Bulolo, cluster 7), which had been not yet been covered by the free LLIN distribution at the time of the survey.[29] However, there was no difference observed for *P. vivax* between sentinel sites and other areas that were covered by the free LLIN distribution. This finding is in line with more marked reductions in incidence and prevalence of *P. falciparum* than *P. vivax* following the first national distribution of LLIN.[76] This may not fully explain the situation in PNG, since a reduction in both *P. falciparum* and *P. vivax* transmission was observed in some areas after LLIN distribution.[51,76] A study from Indonesia showed a positive correlation between the rate of polyclonal infections and annual parasite incidence indicating that polyclonality might provide a complementary gauge of local transmission intensity.[33] In the case of *P. falciparum* in PNG, this measure correlates well with prevalence, however in this and other studies,[77,78] *P. vivax* infections had a high proportion of multiple clones even in areas with decreasing transmission, so polyclonality may be less effective for determining the transmission of the latter species.

The existence of multiple clone infections is believed to be one prerequisite for genetically diverse parasite populations, since it allows the simultaneous transmission of distinct clones that recombine in the mosquito, allowing outcrossing and generating novel genotypes.[12] Genetic diversity was high for both *P. vivax* and *P. falciparum* throughout PNG. Diversity of the *P. vivax* markers *PvMS16* and *Pvmsp1F3* was similar to diversity reported from other endemic settings[42,75,77,78] and similar to previous reports from the north coast of PNG.[42,44,79] Despite high variability in complexity of infection, *P. falciparum* genetic diversity based on the *Pfmsp2* marker was also similar among different geographic areas. However, in some clusters such as the Eastern Highlands (cluster 10) and Morobe: Mumeng area (cluster 8), the genetic diversity of *P. falciparum* was lower indicating much lower transmission or relatively isolated populations in those geographic areas. The levels of diversity on the north coast (Madang and East Sepik) in this study were similar to previous reports from the PNG north coast in 2005–2006 (mean H_e = 0.93)[49] and African parasite populations from high endemicity areas (H_e = 0.96).[80] Moreover, the finding that there was no significant association between parasite prevalence and genetic diversity for either species suggests that diversity is maintained at high levels despite the different levels of malaria endemicity in the different geographic areas. Therefore, substantial reductions in transmission by integrated control efforts will be required to impact parasite diversity and population structure in PNG. The mean MOI however was significantly correlated with parasite genetic diversity (R_s and H_e) for *P. falciparum* consistent with within host complexity as a critical factor for creation of new genotypes,[81] whereas there was a lack of association for *P. vivax* because there was high within host complexity and high levels of diversity across the country. For both species it will therefore be important to investigate diversity using panels of ten or more less diverse genome wide markers such as microsatellite markers[37,82,83] or single nucleotide polymorphisms.[41,84] If chosen carefully, neutral genome wide markers will provide a more accurate estimate of diversity amongst individual parasites and populations, to further investigate the observed variability between regions.

The contrasting molecular epidemiology of *P. vivax* and *P. falciparum* described here indicates that differences in *P. vivax* biology have a major impact on its complexity of infection and diversity, compared with *P. falciparum*. This includes the ability to relapse,[71] the earlier appearance of transmission stages, lower density infections, and a faster acquisition of immunity,[3] the latter two of which may compromise diagnosis and subsequent treatment. *Plasmodium vivax* also has a wider geographical range due to its ability to develop within the mosquito vector at lower temperatures,[4] and at least in part due to a wider vector range in some endemic regions (though in PNG all local malaria vectors transmit both species).[85,86] Of particular note is relapse, which arises from triggering of the dormant liver stage to cause multiple sequential blood-stage infections in the absence of another mosquito bite.[58] These relapses not only cause further illness to the individual, they also provide more opportunities for multiple clones to be co-transmitted, resulting in outcrossing during sexual recombination in the mosquito midgut. Relapse can thus contribute to greater complexity of infection and higher levels of parasite diversity and may also increase gene flow through human movement and help to maintain larger, more stable parasite populations than *P. falciparum*.[23,87,88]

This survey was conducted as a baseline to future surveys monitoring the success of the PNG national malaria control program with the first countrywide LLIN distribution completed in the year of the survey (2008). Since then, *Plasmodium* spp. prevalence in the general population at sentinel sites has decreased from 15.7% in 2009 to below 4.8% in 2011.[76,89] Although the decrease was significant for *P. falciparum* (10.1% in 2009 down to 2.5% in 2011), *P. vivax* parasite rates had a less significant decline (5.9% to 2.4%) and there was a shift to *P. vivax* dominance in some areas.[76] A significant decrease in both species was also noted in malaria cases in sentinel health facilities and the *P. vivax* shift was even less dramatic and up to half of all clinical cases in one sentinel site were due to *P. vivax*. This holds true in other *P. vivax* and *P. falciparum* co-endemic countries such as Ethiopia and Solomon Islands where the relative proportion of *P. vivax* cases has drastically increased.[90–93]

The data also indicate a change in the proportion of *Pfmsp2* alleles over time. The current study demonstrated that 80% of *P. falciparum* isolates had 3D7-type alleles. This family of alleles is highly diverse and dominant in all parasite populations of PNG. However, a 2005 survey conducted on the north coast showed only 68% of clones harbored the 3D7-type alleles[49] and in 1992, it was reported that the *msp2* FC27 allele was at almost equal frequencies with 3D7. A phase 1-2b vaccine (Combination B) trial conducted in the 1990s in the same region of PNG included only the 3D7 allele of *Pfmsp2*. Infections from vaccines had a lower prevalence of the 3D7 allele than the control group, and were more likely to experience clinical episodes with parasites carrying the alternate FC27 allele.[95] A higher prevalence of 3D7 antibody responses after vaccination suggested that acquired immune responses are an important

driver of these changing allele frequencies.[96] These changing allele frequencies would need to be considered in future *Pfmsp2*-based vaccines.

In conclusion, although the prevalence of *P. falciparum* and *P. vivax* was highly variable throughout PNG, the infection complexity was associated with this variation only for *P. falciparum. P. falciparum* genetic diversity varies according to the infection complexity, whereas *P. vivax* infection complexity and diversity remain high throughout PNG. This suggests that *P. vivax* has a higher potential for outcrossing and generation of diversity at a range of transmission levels, and even when *P. falciparum* infections are more common. Even though the current control strategies had a great impact on parasite transmission, superinfection and genetically diverse *P. vivax* parasite populations will be a future challenge for the PNG national malaria control program requiring rigorous and sustained control efforts to reach the goal of elimination from 2025.[5] As the markers used were highly diverse, further studies using less diverse, neutral markers and data from the years after the interventions were introduced are required for dissecting parasite population structure and understand changes in parasite diversity due to intensification of control efforts.

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