

High Genetic Diversity of *Plasmodium falciparum* in the Low-Transmission Setting of the Kingdom of Eswatini

Michelle E. Roh,^{1,2,a} Sofonias K. Tessema,^{3,a} Maxwell Murphy,³ Nomcebo Nhlabathi,⁵ Nomcebo Mkhonta,⁵ Sibonakaliso Vilakati,⁵ Nyasatu Ntshalintshali,⁶ Manik Saini,⁶ Gugu Maphalala,⁷ Anna Chen,³ Jordan Wilhelm,³ Lisa Prach,¹ Roly Gosling,^{1,2} Simon Kunene,⁷ Michelle S. Hsiang,^{1,4,8,b} and Bryan Greenhouse,^{3,9,b}

¹Malaria Elimination Initiative, Institute of Global Health Sciences, ²Department of Epidemiology and Biostatistics, ³Division of HIV, Infectious Diseases, and Global Medicine, Department of Medicine, and ⁴Department of Pediatrics, University of California, San Francisco; ⁵National Malaria Programme, Ministry of Health, Manzini; ⁶Clinton Health Access Initiative, Mbabane, Eswatini, and ⁷Ministry of Health, Mbabane, Eswatini; ⁸Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas; and ⁹Chan Zuckerberg Biohub, San Francisco, California

Background. To better understand transmission dynamics, we characterized *Plasmodium falciparum* genetic diversity in Eswatini, where transmission is low and sustained by importation.

Methods. Twenty-six *P. falciparum* microsatellites were genotyped in 66% of confirmed cases (2014–2016; N = 582). Population and within-host diversity were used to characterize differences between imported and locally acquired infections. Logistic regression was used to assess the added value of diversity metrics to classify imported and local infections beyond epidemiology data alone.

Results. Parasite population in Eswatini was highly diverse (expected heterozygosity [H_E] = 0.75) and complex: 67% polyclonal infections, mean multiplicity of infection (MOI) 2.2, and mean within-host infection fixation index (F_{ws}) 0.84. Imported cases had comparable diversity to local cases but exhibited higher MOI (2.4 vs 2.0; $P = .004$) and lower mean F_{ws} (0.82 vs 0.85; $P = .03$). Addition of MOI and F_{ws} to multivariate analyses did not increase discrimination between imported and local infections.

Conclusions. In contrast to the common perception that *P. falciparum* diversity declines with decreasing transmission intensity, Eswatini isolates exhibited high parasite diversity consistent with high rates of malaria importation and limited local transmission. Estimates of malaria transmission intensity from genetic data need to consider the effect of importation, especially as countries near elimination.

Keywords. malaria; malaria elimination; Eswatini; Swaziland; population genetics; microsatellite genotyping; parasite diversity; transmission intensity.

The Kingdom of Eswatini, formerly the Kingdom of Swaziland, has made significant progress in reducing its malaria burden and is on track to be one of the first sub-Saharan African countries to eliminate malaria. From 2000 to 2010, Eswatini reduced its malaria cases by 94% [1] and has since sustained very low malaria transmission, reporting an average of 1.5 cases per 1000 between 2010 and 2016 [2]. A key challenge in eliminating malaria in Eswatini has been cross-border migration from neighboring, higher-transmission countries, which has resulted in malaria importation and sporadic onward transmission [3–5].

Between 2014 and 2016, a total of 55% of infections were classified as imported [6], mainly from Mozambique (90%) [4, 7].

To complement traditional approaches to surveillance, genotyping of the *Plasmodium falciparum* parasite has become a popular approach to understanding underlying malaria transmission dynamics [8–10]. The current assumption is that as transmission declines, recombination between genetically distinct clones reduces, leading to decreases in genetic diversity [11]. This relationship, sometimes referred to as a “genomic thermometer” for transmission, has been based on numerous studies that have shown that *P. falciparum* parasite populations generally have higher population and within-host diversity in high-transmission settings of sub-Saharan Africa than in lower-transmission settings (eg, Southeast Asia and Latin America) [8, 11–20] and has been considered a useful method for gauging changes in malaria transmission [8, 21, 22]. However, studies of *P. falciparum* parasite diversity in low-transmission settings of sub-Saharan Africa are limited. In these settings, the relationship between transmission intensity and parasite diversity may demonstrate exceptions to this concept, potentially as a result of importation of malaria from neighboring, higher-transmission countries or recent reductions in transmission.

Received 7 January 2019; editorial decision 7 June 2019; accepted 12 June 2019; published online June 13, 2019.

^aM. E. R. and S. K. T. contributed equally to this work.

^bM. S. H. and B. G. contributed equally to this work.

Presented in part: 66th American Society of Tropical Medicine and Hygiene Annual Meeting, 5–9 November, 2017, Baltimore, Maryland.

Correspondence: M. E. Roh, MPH, PhD, Mission Hall UCSF, 550 16th St, San Francisco, CA, 94158 (michelle.roh@ucsf.edu).

The Journal of Infectious Diseases® 2019;220:1346–54

© The Author(s) 2019. Published by Oxford University Press for the Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited. DOI: 10.1093/infdis/jiz305

Here, we evaluate how well the genomic thermometer framework aligns with *P. falciparum* parasite diversity in Eswatini, a low-transmission country challenged by malaria importation from bordering countries with higher malaria transmission, and the utility of parasite diversity metrics to distinguish between imported and locally acquired infections in this setting.

METHODS

Study Site

Eswatini is a small, landlocked country in Southern Africa bordered by South Africa and Mozambique, with an estimated population size of 1.3 million [23]. Approximately 30% of the population live in the eastern half of the country, which is still receptive to malaria. In these areas, malaria transmission is unstable and highly dependent on rainfall and cross-border malaria importation [5, 7, 24].

National Malaria Field Surveillance and Initial Molecular Testing

Malaria is a notifiable disease in Eswatini and all confirmed cases are reported to the national malaria surveillance system [25]. Cases are confirmed by either microscopy or rapid diagnostic test and followed up for investigation, whereby demographics, household GPS coordinates, and travel history are collected. Based on investigation data from the national malaria program, cases are classified as imported if they reported travel to a higher endemic country in the past 8 weeks (excluding travel within the prior week due to a minimum incubation period of 7 days) [26]. If the case resided in a malaria-receptive area, reactive case detection (RACD, ie, focal test and treat using rapid diagnostic tests) is conducted around the household of the index case (ie, the case that triggered the RACD event [27]). Similar epidemiological data are collected from individuals during RACD events and dried blood spots (DBS) are collected from both index and RACD screened cases.

DBS samples underwent molecular testing by loop-mediated isothermal amplification (LAMP) using previously described methods [28]. A second DBS from all index cases and LAMP-positive individuals from RACD was collected for subsequent quantitative polymerase chain reaction (PCR) testing at the University of California, San Francisco (UCSF) using the *varATS* method [29]. Samples were transported at room temperature and stored at -20°C . In both laboratories, DNA was extracted from DBS using the saponin Chelex method [30].

Study Population

Study eligibility included cases identified through Eswatini's national malaria surveillance system between July 2014 and June 2016 (eg, index and RACD cases). Cases had to have a DBS available for genotyping and a parasite density of ≥ 10 parasites/ μL . Ethics approval was granted by the Eswatini Ethics Committee and the UCSF Committee on Human Research.

Microsatellite Genotyping of *P. falciparum*

Samples were genotyped using microsatellite markers at 26 loci distributed across the *P. falciparum* genome [11, 31, 32] (Liu et al, in preparation). The neutrality of these 26 loci were evaluated using the F_{ST} outlier detection method under the infinite alleles model [33]. None of the loci showed evidence of strong balancing or positive selection in 602 samples from 26 sub-Saharan African countries (Liu et al, in preparation). To minimize genotyping errors, strict thresholds were used to call alleles using a semisupervised naive Bayes classifier implemented in microSPAT software [34]. Briefly, the software differentiates true peaks from stutter peaks using a classifier algorithm based on the location and size of locus-specific peak patterns relative to a primary peak. Details of the microSPAT software and user setting parameters are provided in [Supplementary Table 1](#). Capillary electrophoresis peaks were excluded if the probability of being a true allele as determined by the algorithm was $<95\%$. Samples genotyped at ≤ 13 loci were excluded. Data from loci with failed results were treated as missing completely at random and not interpolated with values.

Characterization of Population-level Diversity

Population-level genetic diversity was assessed by expected heterozygosity (H_E), number of alleles per locus (ie, allelic richness), and effective population size (N_e). Values for H_E range from 0 to 1 (0 indicating no diversity and 1 indicating all alleles are different). H_E was calculated for each locus using the equation $H_E = \frac{n}{n-1} [1 - \sum p_i^2]$, where n = the number of samples analyzed, p_i = the allele frequency of the i th allele in the population. Mean H_E was calculated by taking the average of H_E across all loci. The number of unique alleles per locus (A) is an alternative measure of population-level genetic diversity. The N_e was estimated using the infinite alleles model (IAM) and the step-wise mutation model (SMM) as previously described [11]. Genetic differentiation between groups was assessed by comparing H_E differences using the Hedrick G'_{ST} method [35].

To compare Eswatini's *P. falciparum* genetic diversity relative to other malaria-endemic countries, we conducted a literature search of studies that used a similar subset of microsatellite markers and only studies that published expected heterozygosities at each loci were included. Due to the lack of interlaboratory calibration and nonstandardized allele calling practices of capillary-based microsatellite data, estimates of population-level diversity from Eswatini were recalculated by rescaling alleles based on the exact allele calling methods described in each comparator study, using only the subset of microsatellite markers that were genotyped in both the Eswatini and comparator studies.

Characterization of Within-Host Diversity

Within-host diversity was assessed by multiplicity of infection (MOI), proportion of polyclonal infections, and within-host infection fixation index (F_{WS}). MOI was defined as the second highest number of alleles observed in any of the 26 loci, unless

otherwise stated. An infection was considered polyclonal if $MOI > 1$. The F_{WS} metric is a measure of within-host diversity that describes the relationship between the genetic diversity of an individual infection relative to the genetic diversity of the parasite population [36]. A low F_{WS} indicates high within-host diversity relative to the population. F_{WS} was calculated for each sample using the equation $F_{WS} = 1 - \frac{H_w}{H_s}$, where H_w = allele frequency of each unique allele at a particular locus for each individual, and H_s = heterozygosity of the local parasite population. Amplification bias of different-sized amplicons was corrected by estimating preferential bias as a function of amplicon size difference using known controls at known relative densities [34]. Using previously described F_{WS} thresholds [15, 37], samples with $F_{WS} > 0.95$ were considered clonal infections and samples with $F_{WS} \leq 0.70$ were considered highly diverse infections.

Predictive Ability of MOI and F_{WS} to Discriminate Between Imported and Local Cases

Logistic regression was used to assess whether the addition of MOI and mean F_{WS} to epidemiologically parameterized models would predict whether a case was imported or locally acquired. The base epidemiological model included the following predictors: district of residence, season, case detection method (passive vs reactive surveillance), age, sex, and occupation. Model improvement was tested using the log-likelihood X^2 statistic and discriminative ability was assessed by the change in the area under the receiver operating characteristic curve.

Sensitivity Analyses

Sensitivity analyses were conducted to assess whether results obtained from genotyped cases were representative of all cases detected during the 2014–2016 period. Due to missing DBS samples or genotype failures, 298 cases were not genotyped, which may have resulted in a nonrandom sampling of the original case population ($n = 880$). Comparisons between genotyped and nongenotyped cases revealed these 2 groups significantly differed by case detection method (passive vs reactive surveillance), season, district, case classification (imported vs local), sex, and occupation (Supplementary Table 2). To account for this potential source of selection bias, we calculated a propensity score using a logistic regression model [38] with the aforementioned demographic variables to estimate the probability of each case being genotyped. The inverse of these probabilities were used to reweight within-host diversity measures obtained from genotyped individuals to represent the total case population. Other than 3 nongenotyped cases that had a propensity score < 0.17 , density plots showed good overlap of the propensity scores between genotyped and nongenotyped groups (Supplementary Figure 1), suggesting propensity score reweighting was an appropriate approach to obtain generalizable estimates. None of the results were materially affected by this reweighting, therefore the primary results presented below all use the original, unweighted data.

Statistical Tests

All analyses were performed using Stata 14.0 (StataCorp, College Station, TX) and R (version 3.5.0; R Project for Statistical Computing; <http://www.r-project.org/>). Statistical comparisons were conducted using Pearson X^2 test for categorical variables, and Student t tests or Mann-Whitney tests for continuous variables, depending on the degree of normality of underlying distributions. The Spearman rank correlation (ρ) was used to assess the correlation between mean F_{WS} values and MOI. P values $< .05$ were considered statistically significant.

RESULTS

Characteristics of Samples and Genotyping

Between July 2014 and June 2016, 880 *P. falciparum* cases were identified through Eswatini's national malaria surveillance program (Figure 1 and Figure 2). Between 2014–2015 and 2015–2016, Eswatini experienced a 2.6-fold reduction in cases (from 635 to 245 cases). The majority of cases were detected through passive surveillance (82%) and the rest were identified through RACD. Of the cases identified, 666 *P. falciparum*-positive samples were available for genotyping. The mean success rate across all 26 markers was 86% (range, 74–94). Of the 666 genotyped samples, 582 (87%) samples were genotyped successfully at > 13 loci and included in the study. Sixty-six percent of genotyped samples had full coverage of 26 loci. Of the 298/880 cases that were not genotyped, 9 had a parasite density of zero by quantitative PCR (3%) and 75 (25%) had a parasite density below the genotyping threshold (ie, < 10 parasites per μL of blood). The

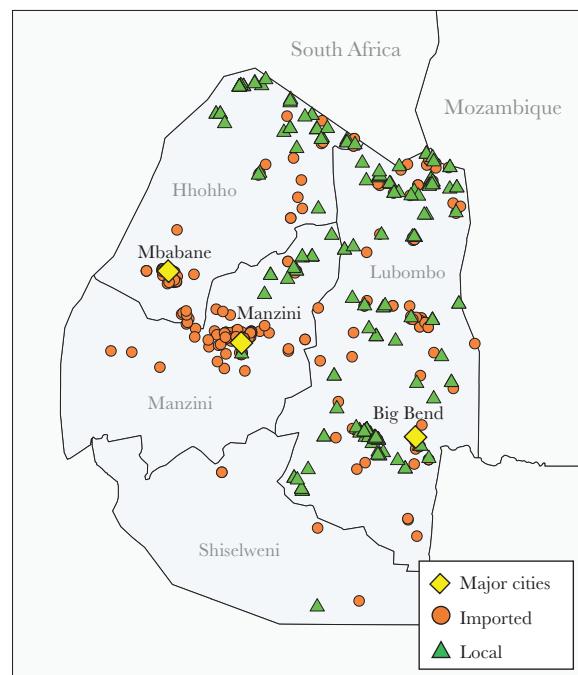


Figure 1. Map of Eswatini cases detected between July 2014 and June 2016. Orange circles represent imported cases and green triangles represent local cases. Yellow diamonds designate major Eswatini cities.

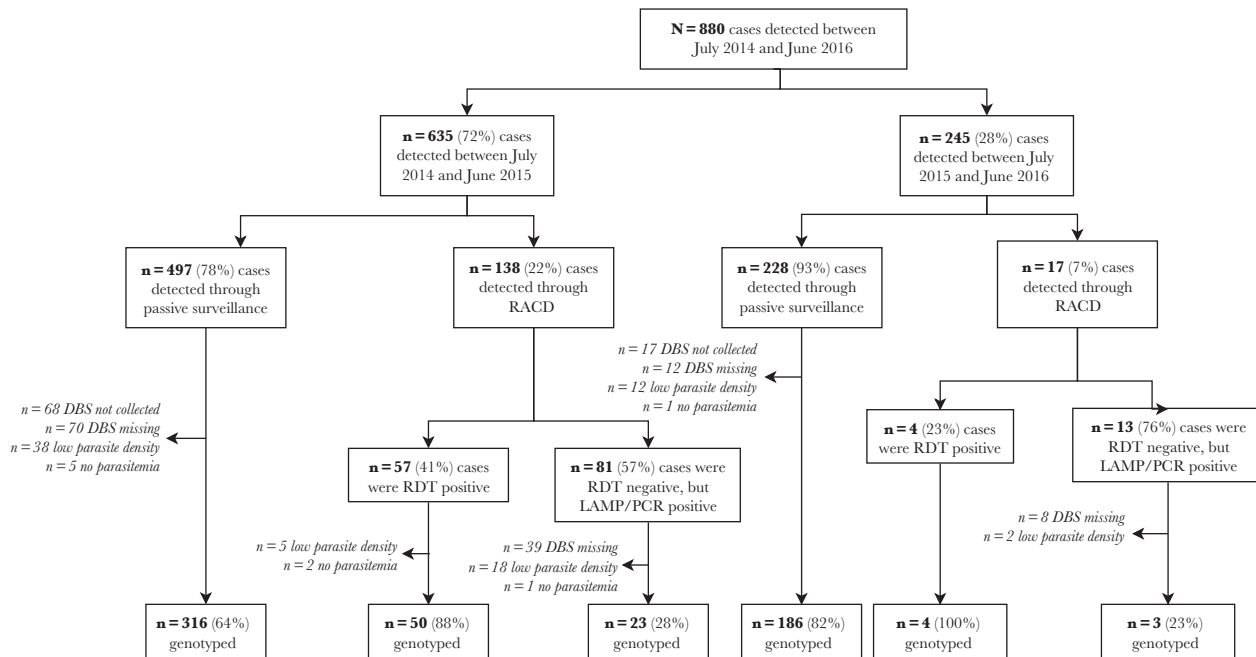


Figure 2. Flow chart of Eswatini samples collected and genotyped between July 2014 and June 2016. Abbreviations: DBS, dried blood spot; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; RACD, reactive case detection; RDT, rapid diagnostic test.

remaining 214 samples were not genotyped due to missing DBS samples (Figure 2). In a sensitivity analysis, no differences in population and within-host diversity were observed between all samples included in the study and the subset of samples with full coverage of all 26 loci (data not shown).

The characteristics of the sample population are presented in Supplementary Table 2. Sixty-two percent of genotyped cases ($n = 359$) were classified as imported by National Malaria Program surveillance officers using self-reported travel history. Of the imported cases that had available country-level travel

data ($n = 188$), 93% reported traveling to Mozambique, with a few cases reporting travel to South Africa ($n = 5$), Zambia ($n = 2$), Zimbabwe ($n = 2$), or Burundi ($n = 2$). Two-hundred and fifteen infections were classified as local infections and only 12 of these were classified as intraport (ie, imported from different areas within Eswatini [5]).

Parasite Populations in Eswatini are Complex and Diverse

Polyclonal infections were found in 66% of cases. Mean MOI was 2.2 (range, 1–5) (Table 1). Mean F_{WS} [15, 36], whereby a low F_{WS}

Table 1. Metrics of Within-Host Diversity in Eswatini by Group

Groups	% Polyclonal (95% CI)	<i>P</i> Value ^b	Mean MOI ± SD (Range)	<i>P</i> Value ^b	Mean F_{WS} ± SD (Range)	<i>P</i> Value ^b
Total (N = 582)	67 (63–70)	...	2.2 ± 1.2 (1.0–7.0)	...	0.84 ± 0.15 (0.44–1.00)	...
Season		.34		.15		.24
June 2014–July 2015 (n = 389)	65 (60–70)		2.2 ± 1.2 (1.0–7.0)		0.84 ± 0.16 (0.44–1.00)	
June 2015–July 2016 (n = 193)	69 (63–76)		2.4 ± 1.2 (1.0–6.0)		0.83 ± 0.15 (0.49–1.00)	
Districts		.032		.30		.11
Hhohho (n = 115)	57 (47–65)		2.1 ± 1.3 (1.0–7.0)		0.87 ± 0.15 (0.49–1.00)	
Lubombo (n = 235)	70 (64–75)		2.1 ± 1.0 (1.0–6.0)		0.83 ± 0.15 (0.47–1.00)	
Manzini (n = 158)	70 (62–76)		2.4 ± 1.3 (1.0–6.0)		0.83 ± 0.16 (0.44–1.00)	
Shiselweni (n = 5)	40 (12–77)		1.8 ± 1.3 (1.0–4.0)		0.86 ± 0.19 (0.58–1.00)	
Passive versus reactive case detection		1.00		.87		.73
Passive (n = 502)	67 (62–71)		2.2 ± 1.2 (1.0–7.0)		0.84 ± 0.15	
Reactive (n = 80)	66 (55–76)		2.2 ± 1.2 (1.0–6.0)		0.83 ± 0.16	
Case classification		.004		.004		.03
Imported (n = 359)	71 (66–76)		2.4 ± 1.3 (1.0–7.0)		0.82 ± 0.16 (0.44–1.00)	
Local ^a (n = 215)	59 (52–65)		2.0 ± 1.1 (1.0–6.0)		0.85 ± 1.15 (0.47–1.00)	

Abbreviations: CI, confidence interval; F_{WS} , within-host diversity relative to that of the population; MOI, multiplicity of infection.

^aLocal infections include indigenous cases ($n = 203$) and intraport (eg, imported from different areas of the country) cases ($n = 12$).

^bAll *P* values were 2-tailed and computed using the χ^2 test for comparing the proportion of polyclonal infections, Poisson regression for MOI, and simple linear regression for F_{WS} .

demonstrates a higher within-host diversity, in the sample population was 0.84 (range, 0.44–1.00) and was negatively correlated with MOI (Supplementary Figure 2). Thirty-eight percent of cases had a F_{WS} value >0.95 (threshold used to indicate clonal infections) and 23% had a $F_{WS} \leq 0.70$ (threshold for infections exhibiting high within-host diversity) [15, 37]. At the population-level, parasite isolates from Eswatini were highly diverse (mean H_E , 0.75; range, 0.52–0.93) (Table 2; Supplementary Table 3) and displayed high allelic richness (mean A, 15.4, SD, 7.1; range, 7–32) (Supplementary Table 4). The estimated N_e was 4717 (95% confidence interval [CI], 2027–10 745) and 11792 (95% CI, 5067–26 863) under IAM and SMM models, respectively.

Despite a 2.6-fold reduction in cases, within-host and population-level diversity were comparable between July–June seasons of 2014–2015 and 2015–2016. Genetic differentiation between seasons was low ($G'_{ST} = 0.026$). No significant differences were observed between districts ($G'_{ST} = 0.0068$), except in Hhohho and Shiselweni districts, where the proportion of monoclonal infections were higher (50/115 or 43% and 3/5 or 60%, respectively) than in Manzini (71/235; 30%) and Lubombo districts (48/158; 30%) ($P = .032$). Within-host, population-level diversity, and genetic structure did not differ between passively versus reactively detected cases (Table 1; $G'_{ST} = 0.003$).

Genetic Diversity of Parasites in Eswatini is Similar to Higher-Transmission Countries

To assess the relative genetic diversity of Eswatini to that of other malaria-endemic countries, estimates derived from Eswatini cases were compared to other genotyping studies using an overlapping subset of microsatellite markers. Our results demonstrate that mean H_E in Eswatini was significantly and consistently higher than those calculated among regions with similarly low malaria transmission intensities (eg, Thailand [18], Vietnam [39], Honduras [40], and Indonesia [41]) and was more

comparable to that of higher-transmission countries (eg, Guinea [14], The Gambia [12], and Mali [13]) (Table 3). Comparison of mean MOI across countries showed that estimates obtained from Eswatini were consistently comparable to estimates obtained from higher-transmission areas including in Ghana [43], Mali [15], and Burkina Faso [15] (Table 4). Among the countries listed in Table 4, the proportion of polyclonal infections was positively correlated with malaria incidence, except in Eswatini, where the proportion of polyclonal infections was higher than all other comparator countries despite having very low malaria incidence.

Imported Cases Are More Complex Than Local Cases

Global measures of population-level and within-host genetic diversity were compared between imported and locally acquired cases, classifications of which were determined by travel history. Population-level diversity was similar between imported and locally acquired cases (Table 2), with little evidence of genetic differentiation ($G'_{ST} = 0.013$). Despite the similarities observed in population-level genetic diversity, infections among locally acquired cases were less complex than imported cases (ie, exhibited lower within-host diversity). A larger proportion of local cases were monoclonal infections compared to imported cases (41% vs 29%; $P = .004$), and had a slightly lower mean MOI (2.0 vs 2.4; $P = .004$) (Table 1; Figure 3). Mean F_{WS} was marginally higher among local cases compared to imported cases (0.85 vs 0.82; $P = .03$). Similarly, a marginally higher proportion of local cases were considered clonal infections by this measure; 44% of local cases had a mean F_{WS} greater than 0.95 compared to 34% of imported cases ($P = .018$). Results from sensitivity analyses that estimated within-host diversity in the original case population ($n = 880$) showed similar patterns of within-host diversity between imported and local cases (Supplementary Table 5).

Table 2. Metrics of Population-Level Genetic Diversity in Eswatini by Group

Groups	Mean $H_E \pm SD$ (Range)	Mean A $\pm SD$ (Range)
Total (N = 582)	0.75 \pm 0.14 (0.52–0.93)	15.4 \pm 7.1 (7.0–32.0)
Season		
June 2014–July 2015 (n = 389)	0.75 \pm 0.14 (0.54–0.93)	15.0 \pm 6.9 (6.0–30.0)
June 2015–July 2016 (n = 193)	0.74 \pm 0.16 (0.46–0.94)	12.4 \pm 6.6 (4.0–27.0)
Districts		
Hhohho (n = 115)	0.75 \pm 0.14 (0.50–0.92)	12.0 \pm 5.7 (4.0–22.0)
Lubombo (n = 235)	0.75 \pm 0.14 (0.52–0.93)	13.9 \pm 6.5 (6.0–29.0)
Manzini (n = 158)	0.75 \pm 0.15 (0.50–0.93)	12.7 \pm 6.3 (4.0–26.0)
Shiselweni (n = 5)	0.72 \pm 0.19 (0.34–0.97)	3.2 \pm 1.3 (2.0–6.0)
Passive versus reactive case detection		
Passive (n = 502)	0.75 \pm 0.14 (0.52–0.93)	15.3 \pm 7.1 (7.0–31.0)
RACD (n = 80)	0.75 \pm 0.14 (0.51–0.94)	10.8 \pm 6.1 (4.0–27.0)
Case classification		
Imported (n = 359)	0.75 \pm 0.15 (0.51–0.94)	14.5 \pm 7.0 (5.0–30.0)
Local ^a (n = 215)	0.75 \pm 0.14 (0.49–0.91)	13.2 \pm 6.2 (4.0–25.0)

Abbreviations: A, number of unique alleles per locus; H_E , expected heterozygosity; RACD, reactive case detection.

^aLocal infections include intraportected infections (n = 12) and indigenous cases (n = 203).

Table 3. Comparison of Mean H_E Observed in Eswatini Isolates With Previously Published Studies

Country	Year	Annual Confirmed Cases per 1000 ^a	H _E		No. of Loci Compared	P Value ^c	Reference, Publication Year
			Country	Eswatini ^b			
Nicaragua	2011	0.1	0.47	0.87	3	.003	Larrañaga et al, 2013 [40]
Thailand	2002–2007	0.5	0.65	0.87	6	.01	Pumpaibool et al, 2009 [18]
Vietnam	1996–1997	0.75	0.76	0.87	6	.028	Ferreira et al, 2002 [39]
Honduras	2011	1	0.31	0.88	3	.017	Larrañaga et al, 2013 [40]
Indonesia (Papua)	2011–2014	1.5	0.69	0.87	8	.02	Pava et al, 2017 [41]
Peru	2003–2007	3	0.53	0.91	3	.078	Branch et al, 2011 [42]
Republic of Guinea	2011	8	0.85	0.87	7	.17	Murray et al, 2016 [14]
The Gambia	2005–2009	39	0.83	0.87	7	.06	Mobegi et al, 2012 [12]
Mali	2012–2015	70	0.84	0.86	5	.42	Nabet et al, 2016 [13]

Abbreviation: H_E, expected heterozygosity.

^aEstimates from the World Health Organization Malaria Report. Year 2000 incidence estimates are reported for studies conducted before 2000. Malaria incidence in Eswatini between 2014 and 2016 was estimated at 1 case per 1000 person-years.

^bEswatini H_E recalculated using only identical loci and the same threshold for allele calling in the comparison study.

^cP value generated using a 2-tailed Student *t* test comparing mean H_E values across the subset of loci compared between studies.

Table 4. Comparison of Polyclonal Infections and MOI of Eswatini With Previously Published Studies

Country	Year	Annual Confirmed Cases per 1000 ^a	Mean MOI	Polyclonal Infections, %	Reference, Publication Year
Thailand	2002–2007	0.5	...	28	Ferreira et al, 2002 [39]
Eswatini	2014–2016	1	2.2 ^b (2.7 ^c)	67 ^b (74 ^c)	This study
Indonesia (Papua)	2011–2014	1.5	1.2 ^c	17	Pava et al, 2017 [41]
Peru	2003–2007	3	...	34	Branch et al, 2011 [42]
Ghana	2003–2004	28	2.3 ^d	...	Agyeman-Budu, 2013 [43]
Mali	2012–2015	70	2.6 ^c	59	Auburn et al, 2012 [15]
Burkina Faso	2012	248	3.0 ^c	...	Auburn et al, 2012 [15]
Southern Mozambique (Maputo Province)	1997–1999	312	1.8 ^d	49	Mayor et al, 2003 [44]

Abbreviation: MOI, multiplicity of infection.

^aEstimates from the World Health Organization Malaria Report. Year 2000 incidence estimates are reported for studies conducted before 2000. Malaria incidence in Eswatini between 2014 and 2016 was estimated at 1 cases per 1000 person-years.

^bCalculated from the second-highest MOI.

^cCalculated from maximum alleles found at any 1 microsatellite.

^dCalculated from *m*sp-2 typing.

Inclusion of MOI and F_{ws} Does Not Meaningfully Improve Discrimination Between Imported and Local Cases

Given the marginally, but statistically significant, differences in MOI and F_{ws} between local and imported cases, we evaluated whether these metrics could predict whether a case was local versus imported, because travel history data are not always available or accurate. Compared to a base model that included district of residence, season, passively versus actively detected case, age, sex, and occupation to predict malaria importation, the model that included F_{ws} and MOI marginally improved the goodness of fit (log-likelihood X^2 statistic difference = +8.17; $P = .017$), but had negligible effects on improving the discrimination between imported and local cases (area under the curve difference = +0.002; $P = .6$) (Supplementary Figure 3).

DISCUSSION

Despite sustaining low malaria transmission for nearly a decade, our study demonstrated that malaria in Eswatini is largely

characterized by polyclonal, complex *P. falciparum* infections and a markedly diverse parasite population. This evidence was generated by genotyping two-thirds of all documented, symptomatic and asymptomatic *P. falciparum* infections in the country over the span of two years. The genetic diversity and large effective population size reveal a *P. falciparum* population similar to those observed among higher-transmission regions of sub-Saharan Africa [12–15, 44]. Imported and local cases had similar levels of population-level diversity and though MOI was higher and F_{ws} was lower among imported cases, the magnitude of these differences did not allow for accurate discrimination between these 2 groups. This suggests that parasite diversity in Eswatini is largely dominated by its cross-border migration from neighboring, higher-transmission countries (eg, Mozambique). However, future molecular surveillance studies of parasite diversity in Mozambique should be conducted to further confirm these findings.

These data present an important caveat to the genomic thermometer framework; namely, the assumption that as

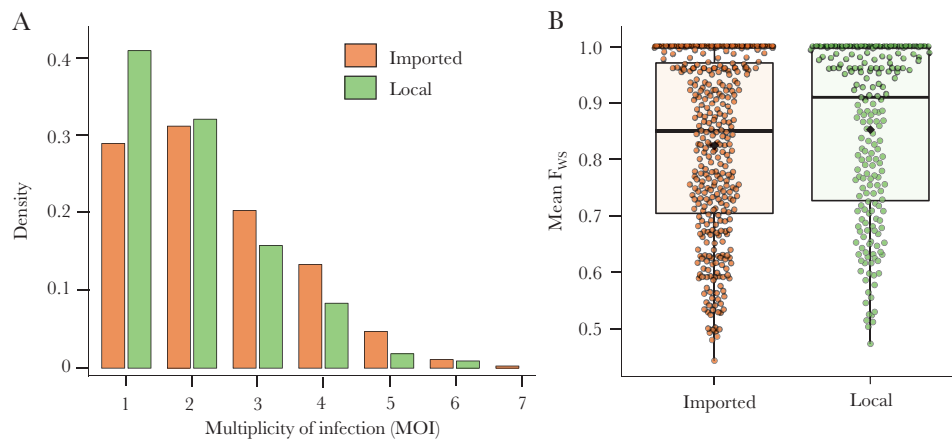


Figure 3. Distribution of multiplicity of infection (MOI) (A) and mean within-host infection fixation index (F_{ws}) (B) by imported and locally acquired cases. Orange indicates the distribution of MOI and F_{ws} among imported cases and green indicates distribution among locally acquired cases. B, Black horizontal line indicates the median, diamonds the mean, and the upper and lower bounds of the box indicate the 25th and 75th quartiles.

malaria reaches very low levels of transmission, *P. falciparum* populations are expected to consistently exhibit low parasite diversity [8, 11, 16–20]. Although a monotonic relationship between diversity and transmission has been observed in low-transmission settings of Southeast Asia or Latin America, where importation of malaria from higher-transmission areas is uncommon [11, 16, 17, 20, 22, 45] and/or where parasites are imported from areas with low genetic diversity [19], the genetic thermometer framework fails to accurately represent countries like Eswatini. In the low-transmission setting of Eswatini, we observed an inverse relationship between genetic diversity and transmission intensity, which is consistent with the country's malaria epidemiological patterns. It appears that substantial human mobility from neighboring higher-transmission countries [3, 4, 7] has resulted in the frequent importation of highly diverse parasites [46, 47]. This inverse relationship may become more evident as more genotyping studies from other sub-Saharan countries with similar epidemiology become available.

In this setting, further decreases in local transmission may have actually resulted in increases in parasite diversity due to: (1) an increase in the proportion of imported infections, which tend to have higher parasite diversity, and (2) shorter local transmission. Shorter chains would be expected to increase population diversity, due to fewer related parasites circulating, and to increase within-host diversity, as decreases from imported infections likely occur with each generation of local transmission (in the absence of superinfection). As more sub-Saharan African countries with similar challenges move towards malaria elimination, this apparent paradox may become the rule rather than the exception and, as such, these metrics of diversity may not be the most appropriate methods to assess changes in transmission intensity in these settings.

Malaria importation remains a significant barrier for eliminating malaria in many countries and is further complicated

by the difficulty in accurately distinguishing between imported and locally acquired infections. In Eswatini, imported and local infections had similar levels of population-level genetic diversity, with little evidence of genetic differentiation between groups. Imported cases displayed significantly higher within-host diversity compared to local cases, but these differences were minimal and likely due to the limited length of local transmission chains, as described above. As a result, these differences were too small to discriminate between imported and local infections.

One particular strength of the study was its large sample size, allowing for the most extensive and dense capture of genotyped cases from any low-transmission setting to date. Though the study was able to successfully genotype 66% of all documented cases and included sensitivity analyses which suggested that the estimated within-host diversity metrics were representative of the total case population ($n = 880$), our results may not be generalizable to infections not detected through the malaria surveillance system (eg. asymptomatic infections). However, the study was able to evaluate 80 cases captured through RACD, which demonstrated no differences with symptomatic cases. Second, though imported and local infections were classified using detailed travel history information and demographic factors by trained malaria surveillance officers and study team members, it is possible that cases may have been misclassified and may have biased the discriminatory capacity of MOI towards the null. Third, in contrast to comparisons of H_E , which were recalculated and standardized between studies, comparisons of within-host genetic diversity between Eswatini and other studies may be limited in interpretability as different loci and methods were used to calculate MOI and should be interpreted as general trends without focusing on individual comparisons.

In conclusion, these findings demonstrate that *P. falciparum* genetic diversity and transmission intensity do not always follow a simple, monotonic relationship and suggest that

the *P. falciparum* parasite population in an eliminating country with strong connectivity to higher-transmission countries is likely to be highly diverse. Thus, a more nuanced approach must be taken when interpreting the results from genetic analyses, particularly as a means of monitoring and evaluating the impact of malaria interventions in these settings. In countries such as Eswatini, where transmission is largely dominated by the importation of cases from higher-transmission countries, traditional metrics derived from genotyping data, such as MOI, may not be informative. In these settings, detailed characterization of *P. falciparum* transmission, or interruption thereof, may be better achieved through a more comprehensive analysis of transmission networks.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. The authors thank the participants for contributing their samples to the study; the health worker staff who performed sample and data collection; and the Eswatini National Malaria Program and the Clinton Health Access Initiative for their support in data collection.

Disclaimer. The funders of the study had no role in the study design, data collection, data interpretation, or writing of the manuscript. The authors had final responsibility for the decision to submit for publication.

Financial support. This work was supported by the Bill and Melinda Gates Foundation (grant number OPP1132226); the National Institutes of Health/National Institute of Allergy and Infectious Diseases (grant number AI101012); and Burroughs Wellcome Fund/American Society of Tropical Medicine and Hygiene (grant number A120079); surveillance activities were also supported by the Eswatini Ministry of Health, partly through funding from the Global Fund to Fight AIDS, Tuberculosis, and Malaria.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. World Health Organization. World malaria report 2016. Geneva, Switzerland: World Health Organization, 2017.
2. World Health Organization. World malaria report 2017. Country profiles: Swaziland. Geneva, Switzerland: World Health Organization, 2018.

3. Kunene S, Phillips AA, Gosling RD, Kandula D, Novotny JM. A national policy for malaria elimination in Swaziland: a first for sub-Saharan Africa. *Malar J* 2011; 10:313.
4. Koita K, Novotny J, Kunene S, et al. Targeting imported malaria through social networks: a potential strategy for malaria elimination in Swaziland. *Malar J* 2013; 12:219.
5. Cohen JM, Dlamini S, Novotny JM, Kandula D, Kunene S, Tatem AJ. Rapid case-based mapping of seasonal malaria transmission risk for strategic elimination planning in Swaziland. *Malar J* 2013; 12:61.
6. Ministry of Health, Swaziland. National Malaria Control Programme. Malaria Programme mid-term review (MTR) report. Mbabane, Swaziland: Ministry of Health, 2017
7. Tejedor-Garavito N, Dlamini N, Pindolia D, et al. Travel patterns and demographic characteristics of malaria cases in Swaziland, 2010–2014. *Malar J* 2017; 16:359.
8. Auburn S, Barry AE. Dissecting malaria biology and epidemiology using population genetics and genomics. *Int J Parasitol* 2017; 47:77–85.
9. Daniels RE, Schaffner SF, Wenger EA, et al. Modeling malaria genomics reveals transmission decline and rebound in Senegal. *Proc Natl Acad Sci U S A* 2015; 112:7067–72.
10. Koepfli C, Mueller I. Malaria epidemiology at the clone level. *Trends Parasitol* 2017; 33:974–85.
11. Anderson TJ, Haubold B, Williams JT, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* 2000; 17:1467–82.
12. Mobegi VA, Loua KM, Ahouidi AD, et al. Population genetic structure of *Plasmodium falciparum* across a region of diverse endemicity in West Africa. *Malar J* 2012; 11:223.
13. Nabet C, Doumbo S, Jeddi F, et al. Genetic diversity of *Plasmodium falciparum* in human malaria cases in Mali. *Malar J* 2016; 15:353.
14. Murray L, Mobegi VA, Duffy CW, et al. Microsatellite genotyping and genome-wide single nucleotide polymorphism-based indices of *Plasmodium falciparum* diversity within clinical infections. *Malar J* 2016; 15:275.
15. Auburn S, Campino S, Miotto O, et al. Characterization of within-host *Plasmodium falciparum* diversity using next-generation sequence data. *PLoS One* 2012; 7:e32891.
16. Anthony TG, Conway DJ, Cox-Singh J, et al. Fragmented population structure of *Plasmodium falciparum* in a region of declining endemicity. *J Infect Dis* 2005; 191:1558–64.
17. Iwagami M, Rivera PT, Villacorte EA, et al. Genetic diversity and population structure of *Plasmodium falciparum* in the Philippines. *Malar J* 2009; 8:96.
18. Pumpaibool T, Arnathau C, Durand P, et al. Genetic diversity and population structure of *Plasmodium falciparum* in Thailand, a low transmission country. *Malar J* 2009; 8:155.

19. Gray KA, Dowd S, Bain L, et al. Population genetics of *Plasmodium falciparum* and *Plasmodium vivax* and asymptomatic malaria in Temotu Province, Solomon Islands. *Malar J* **2013**; 12:429.
20. Noviyanti R, Coutrier F, Utami RA, et al. Contrasting transmission dynamics of co-endemic *Plasmodium vivax* and *P. falciparum*: implications for malaria control and elimination. *PLoS Negl Trop Dis* **2015**; 9:e0003739.
21. Conway DJ. Molecular epidemiology of malaria. *Clin Microbiol Rev* **2007**; 20:188–204.
22. Volkman SK, Neafsey DE, Schaffner SF, Park DJ, Wirth DF. Harnessing genomics and genome biology to understand malaria biology. *Nat Rev Genet* **2012**; 13:315–28.
23. World Health Organization. Eswatini. <http://www.who.int/countries/swz/en/>. Accessed 20 June 2019.
24. Chuang TW, Soble A, Ntshalintshali N, et al. Assessment of climate-driven variations in malaria incidence in Swaziland: toward malaria elimination. *Malar J* **2017**; 16:232.
25. World Health Organization. Malaria elimination by 2020 is possible in Swaziland. <http://www.afro.who.int/news/malaria-elimination-2020-possible-swaziland>. Accessed 20 June 2019.
26. World Health Organization. Disease surveillance for malaria control: operational manual. Geneva, Switzerland: World Health Organization, **2012**.
27. World Health Organization. A framework for malaria elimination. Geneva, Switzerland: World Health Organization, **2017**.
28. Dlamini N, Hsiang MS, Ntshalintshali N, et al. Low-quality housing is associated with increased risk of malaria infection: a national population-based study from the low transmission setting of Swaziland. *Open Forum Infect Dis* **2017**; 4:ofx071.
29. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med* **2015**; 12:e1001788.
30. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* **1995**; 52:565–8.
31. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. *Parasitology* **1999**; 119:113–25.
32. Pringle JC, Tessema S, Wesolowski A, et al. Genetic evidence of focal *Plasmodium falciparum* transmission in a pre-elimination setting in Southern Province, Zambia. *J Infect Dis* **2018**; 219:1254–63.
33. Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G. LOSITAN: a workbench to detect molecular adaptation based on a FST-outlier method. *BMC Bioinformatics* **2008**; 9:323.
34. Murphy M. Greenhouse-Lab/MicroSPAT: initial release. *Zenodo* **2018**; doi: 10.5281/zenodo.1469126
35. Hedrick PW. A standardized genetic differentiation measure. *Evolution* **2005**; 59:1633–8.
36. Manske M, Miotto O, Campino S, et al. Analysis of *Plasmodium falciparum* diversity in natural infections by deep sequencing. *Nature* **2012**; 487:375–9.
37. Mobegi VA, Duffy CW, Amambua-Ngwa A, et al. Genome-wide analysis of selection on the malaria parasite *Plasmodium falciparum* in West African populations of differing infection endemicity. *Mol Biol Evol* **2014**; 31:1490–9.
38. Seaman SR, White IR. Review of inverse probability weighting for dealing with missing data. *Stat Methods Med Res* **2013**; 22:278–95.
39. Ferreira MU, Nair S, Hyunh TV, Kawamoto F, Anderson TJ. Microsatellite characterization of *Plasmodium falciparum* from cerebral and uncomplicated malaria patients in Southern Vietnam. *J Clin Microbiol* **2002**; 40:1854–7.
40. Larrañaga N, Mejía RE, Hormaza JJ, Montoya A, Soto A, Fontecha GA. Genetic structure of *Plasmodium falciparum* populations across the Honduras-Nicaragua border. *Malar J* **2013**; 12:354.
41. Pava Z, Handayani I, Trianty L, et al. Passively versus actively detected malaria: similar genetic diversity but different complexity of infection. *Am J Trop Med Hyg* **2017**; 97:1788–96.
42. Branch OH, Sutton PL, Barnes C, et al. *Plasmodium falciparum* genetic diversity maintained and amplified over 5 years of a low transmission endemic in the Peruvian Amazon. *Mol Biol Evol* **2011**; 28:1973–86.
43. Agyeman-Budu A, Brown C, Adjei G, et al. Trends in multiplicity of *Plasmodium falciparum* infections among asymptomatic residents in the middle belt of Ghana. *Malar J* **2013**; 12:22.
44. Mayor A, Saute F, Aponte JJ, et al. *Plasmodium falciparum* multiple infections in Mozambique, its relation to other malariological indices and to prospective risk of malaria morbidity. *Trop Med Int Health* **2003**; 8:3–11.
45. Escalante AA, Ferreira MU, Vinetz JM, et al. Malaria molecular epidemiology: lessons from the international centers of excellence for malaria research network. *Am J Trop Med Hyg* **2015**; 93:79–86.
46. Reiner RC Jr, Le Menach A, Kunene S, et al. Mapping residual transmission for malaria elimination. *Elife* **2015**; 4:pii: e09520.
47. Churcher TS, Cohen JM, Novotny J, Ntshalintshali N, Kunene S, Cauchemez S. Public health. Measuring the path toward malaria elimination. *Science* **2014**; 344:1230–2.