High Genetic Diversity of \textit{Plasmodium falciparum} in the Low-Transmission Setting of the Kingdom of Eswatini

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\textbf{Background.} To better understand transmission dynamics, we characterized \textit{Plasmodium falciparum} genetic diversity in Eswatini, where transmission is low and sustained by importation.

\textbf{Methods.} Twenty-six \textit{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.

\textbf{Results.} Parasite population in Eswatini was highly diverse (expected heterozygosity [HE] = 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; \textit{P} = .004) and lower mean F_{WS} (0.82 vs 0.85; \textit{P} = .03). Addition of MOI and F_{WS} to multivariate analyses did not increase discrimination between imported and local infections.

\textbf{Conclusions.} In contrast to the common perception that \textit{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.

\textbf{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 \textit{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 \textit{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 \textit{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.
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 naïve 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 $H_{E} = \frac{\sum_{i=1}^{n} p_i^2}{n}$, where $n$ is the number of samples analyzed, $p_i$ is 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 locus 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 resoring 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 $F_{\text{WS}}$ 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 $F_{\text{WS}}$ indicates high within-host diversity relative to the population. $F_{\text{WS}}$ was calculated for each sample using the equation $F_{\text{WS}} = 1 - H_{w}/H_{s}$, 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 $F_{\text{WS}}$ thresholds [15, 37], samples with $F_{\text{WS}}$ >0.95 were considered clonal infections and samples with $F_{\text{WS}}$ ≤0.70 were considered highly diverse infections.

**Predictive Ability of MOI and $F_{\text{WS}}$ to Discriminate Between Imported and Local Cases**

Logistic regression was used to assess whether the addition of MOI and mean $F_{\text{WS}}$ 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 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 $\chi^{2}$ 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 ($\rho$) was used to assess the correlation between mean $F_{\text{WS}}$ 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
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 intraported (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 FWS [15, 36], whereby a low FWS

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

| Groups               | % Polyclonal (95% CI) | PValueb | Mean MOI ± SD (Range) | PValueb | Mean FWS ± SD (Range) | PValueb |
|----------------------|-----------------------|---------|-----------------------|---------|-----------------------|---------|
| 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  | 65 (60–70)            | 2.2 ± 1.2 (1.0–7.0) | 0.84 ± 0.16 (0.44–1.00) | .30     | .11       |         |
| June 2016–July 2016  | 69 (63–78)            | 2.4 ± 1.2 (1.0–6.0) | 0.83 ± 0.15 (0.49–1.00) | .032    | .11       |         |
| Districts            |                       | .032    |                       | .004    | .03       |         |
| Hhohho (n = 115)     | 57 (47–65)            | 2.1 ± 1.3 (1.0–7.0) | 0.87 ± 0.15 (0.49–1.00) | .30     | .11       |         |
| Lubombo (n = 235)    | 70 (64–75)            | 2.1 ± 1.0 (1.0–6.0) | 0.83 ± 0.15 (0.47–1.00) | .032    | .03       |         |
| Manzini (n = 158)    | 70 (62–76)            | 2.4 ± 1.3 (1.0–6.0) | 0.83 ± 0.16 (0.44–1.00) | .004    | .004      | .03     |
| Shiselweni (n = 6)   | 40 (12–77)            | 1.8 ± 1.3 (1.0–4.0) | 0.86 ± 0.19 (0.58–1.00) | .73     | .73       |         |
| 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 | .004    | .004      | .03     |
| Reactive (n = 80)    | 66 (58–76)            | 2.2 ± 1.2 (1.0–6.0) | 0.83 ± 0.16 (0.49–1.00) | .004    | .004      | .03     |
| 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) | .73     | .73       |         |
| Local* (n = 215)     | 59 (52–65)            | 2.0 ± 1.1 (1.0–6.0) | 0.85 ± 1.15 (0.47–1.00) | .73     | .73       |         |

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

*Local infections include indigenous cases (n = 203) and intraported (eg, imported from different areas of the country) cases (n = 12).

bAll P values were 2-tailed and computed using the X^2 test for comparing the proportion of polyclonal infections, Poisson regression for MOI, and simple linear regression for FWS.
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 $F_{WS}$ ≤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 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 $H_e$ ± SD (Range) | Mean $A$ ± SD (Range) |
|----------------|-------------------------|-----------------------|
| Total ($N = 582$) | 0.75 ± 0.14 (0.52–0.93) | 15.4 ± 7.1 (7.0–32.0) |
| Season         |                         |                       |
| June 2014–July 2015 ($n = 389$) | 0.75 ± 0.14 (0.54–0.93) | 15.0 ± 6.9 (6.0–30.0) |
| June 2015–July 2016 ($n = 193$) | 0.74 ± 0.16 (0.48–0.94) | 12.4 ± 6.6 (4.0–27.0) |
| Districts      |                         |                       |
| Hhohho ($n = 115$) | 0.75 ± 0.14 (0.50–0.92) | 12.0 ± 5.7 (4.0–22.0) |
| Lubombo ($n = 235$) | 0.75 ± 0.14 (0.52–0.93) | 13.9 ± 6.5 (6.0–29.0) |
| Manzini ($n = 158$) | 0.75 ± 0.15 (0.50–0.93) | 12.7 ± 6.3 (4.0–26.0) |
| Shiselweni ($n = 5$) | 0.72 ± 0.19 (0.34–0.97) | 3.2 ± 1.3 (2.0–6.0) |
| Passive versus reactive case detection |                         |                       |
| Passive ($n = 502$) | 0.75 ± 0.14 (0.52–0.93) | 15.3 ± 7.1 (7.0–31.0) |
| RACD ($n = 80$) | 0.75 ± 0.14 (0.51–0.94) | 10.8 ± 6.1 (4.0–27.0) |
| Case classification |                         |                       |
| Imported ($n = 359$) | 0.75 ± 0.15 (0.51–0.94) | 14.5 ± 7.0 (5.0–30.0) |
| Local* ($n = 215$) | 0.75 ± 0.14 (0.49–0.91) | 13.2 ± 6.2 (4.0–25.0) |

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

*Local infections include imported infections ($n = 12$) and indigenous cases ($n = 203$).
Inclusion of MOI and FWS Does Not Meaningfully Improve Discrimination Between Imported and Local Cases

Given the marginally, but statistically significant, differences in MOI and FWS 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 FWS and MOI marginally improved the goodness of fit (log-likelihood $\chi^2$ 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 FWS 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
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 (e.g., 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*<sub>W</sub>, 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 \textit{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 \textit{P. falciparum} transmission, or interruption thereof, may be better achieved through a more comprehensive analysis of transmission networks.

\textbf{Supplementary Data}

Supplementary materials are available at \textit{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.

\textbf{Notes}

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