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Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space

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Abstract

Within the vertebrate host, infections of a malaria parasite (*Plasmodium*) could include a single genotype of cells (single-clone infections) or two to several genotypes (multiclone infections). Clonal diversity of infection plays an important role in the biology of the parasite, including its life history, virulence, and transmission. We determined the clonal diversity of *Plasmodium mexicanum*, a lizard malaria parasite at a study region in northern California, using variable microsatellite markers, the first such study for any malaria parasite of lizards or birds (the most common hosts for *Plasmodium* species). Multiclonal infections are common (50–88% of infections among samples), and measures of genetic diversity for the metapopulation (expected heterozygosity, number of alleles per locus, allele length variation, and effective population size) all indicated a substantial overall genetic diversity. Comparing years with high prevalence (1996–1998 = 25–32% lizards infected), and years with low prevalence (2001–2005 = 6–12%) found fewer alleles in samples taken from the low-prevalence years, but no reduction in overall diversity (H = 0.64–0.90 among loci). In most cases, rare alleles appeared to be lost as prevalence declined. For sites chronically experiencing low transmission intensity (prevalence ~1%), overall diversity was also high (H = 0.79–0.91), but there were fewer multiclonal infections. Theory predicts an apparent excess in expected heterozygosity follows a genetic bottleneck. Evidence for such a distortion in genetic diversity was observed after the drop in parasite prevalence under the infinite alleles mutation model but not for the stepwise mutation model. The results are similar to those reported for the human malaria parasite, *Plasmodium falciparum*, worldwide, and support the conclusion that malaria parasites maintain high genetic diversity in host populations despite the potential for loss in alleles during the transmission cycle or during periods/locations when transmission intensity is low.

Keywords: clonal diversity, lizard malaria, microsatellites, *Plasmodium*, *Plasmodium mexicanum*, population structure

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Introduction

Within their vertebrate hosts, malaria parasites (*Plasmodium*) replicate asexually as clones of haploid cells, and each infection may consist a single genotype of cells (single-clone infection), or several to many genotypes (multiclone infection) (Babiker *et al*. 1999). Theory argues that the clonal diversity of infections should play a central role in the biology of microparasites such as *Plasmodium* (Griffin & West 2002; Foster 2005), but data have long been lacking to test this view (Read & Taylor 2001). In the case of *Plasmodium*, though, evidence is growing that genetic diversity is indeed important for competitive interactions among clones (deRoode *et al*. 2005), virulence (Smith *et al*. 1999; Read & Taylor 2001), facilitation (positive interaction) (Gilbert *et al*. 1998), premunition (prevention against superinfection) (Smith *et al*. 1999; Vardo *et al*. 2007), and

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degree of inbreeding and optimal sex allocation (Read *et al*. 1992). Data on clonal diversity among infections also provides insight into population structure (Konaté *et al*. 1999; Awadalla *et al*. 2001) and the transmission dynamics within and among sites (Paul *et al*. 1998; Anderson *et al*. 2000a; Ferdig & Su 2000). Thus, assessing the clonal diversity of *Plasmodium*, both within and among infections, is necessary for any understanding of the ecology of these diverse parasites.

The number of clones within malaria infections must depend in part on the prevailing genetic diversity of the parasites among all infections (the parasite's metapopulation). Tibayrenc *et al*. (1991) and Tibayrenc & Ayala (2002) noted that, despite the obligate sexual cycle in *Plasmodium* parasites, *Plasmodium falciparum* in human populations at some locations may have little clonal diversity or even a uniclonal population structure. Malaria parasites could lose genetic diversity if not all clones are successful during the transmission cycle, or if competition within infections excludes clones. During times or at locations experiencing low transmission intensity, overall clonal diversity could decline. Empirical studies on clonal structure of *Plasmodium* infections of humans reveal multiclonal infections are common at locations where transmission is intense (many vectors biting), with lower clonal diversity where transmission is rare (Paul *et al*. 1995, 1998; Anderson *et al*. 1999, 2000; Babiker *et al*. 1999, 2000; Ferreira *et al*. 2002; Abdel-Muhsin *et al*. 2003; Cui *et al*. 2003; Durand *et al*. 2003; Imwong *et al*. 2006; Zakeri *et al*. 2006). Although these results indicate a substantial range of genetic structure among geographical locations, the results are confounded by rapid movement of humans among geographical sites, widespread bed net use, and selective sweeps experienced by human malaria parasites caused by antiparasite drug use (Nair *et al*. 2003). Data on clonal diversity for malaria parasites of nonhuman vertebrate hosts (reptiles, birds, and nonhuman mammals) are not available. These systems, though, may provide a more clear picture of how the life cycle itself may determine the genetic structure of the parasites.

Here we use recently characterized variable microsatellite loci for the lizard malaria parasite, *Plasmodium mexicanum* (Schall & Vardo 2007), to determine the clonal diversity both within and among infections over years and among sites. This system is particularly useful for such studies because prevalence of *P. mexicanum* in its natural lizard host, the western fence lizard, *Sceloporus occidentalis*, has waxed and waned over a 28-year period at our long-term study location in northern California, USA. Also, there are sites at the study location where infection prevalence has been chronically high or low; that is, even as overall prevalence has changed over the years, some sites always had the highest prevalence and some maintained consistently much lower prevalence. Our goals were (i) to determine the distribution of number of clones among infections of *P. mexicanum* using microsatellite markers. These data will reveal if vector-borne parasites such as *Plasmodium* can maintain a high clonal diversity in a host with little host mixing among geographical locations. (ii) To compare clonal diversity of *P. mexicanum* for a period of years when prevalence of the parasite was high vs. another period when prevalence was low. Such sudden drops in prevalence appear common for malaria parasites of both human and nonhuman vertebrate hosts (Schall & Marghoob 1995; Babiker *et al*. 2000), so the data can reveal if these changes in prevalence are associated with a reduction in genetic diversity for the parasite metapopulation. (iii) To examine clonal diversity at sites with consistently low prevalence to determine if long-term weak transmission intensity will result in very low clonal diversity for the parasite. We then compare our results with those reported for human malaria parasites over a worldwide distribution. Thus, we can compare the clonal diversity seen in human malaria parasites, systems that have been greatly disturbed by population mixing and interventions to reduce transmission and infection, with an undisturbed system in a nonhuman host.

Materials and methods

Study areas

The study area was a 2169-ha tract of grass-oak woodland at the Hopland Research and Extension Center ('Hopland') 6.4 km northeast from the town of Hopland in southern Mendocino County, California (Schall 1996). The climate at Hopland is hot and dry in the summer, and cool and rainy in winter, with transmission of *Plasmodium mexicanum* ceasing during the cool months. *Plasmodium mexicanum* infects only the fence lizards at the site, and two species of sandflies (Psychodidae) are the insect vectors (*Lutzomyia vexator* and *Lutzomyia stewartii*) (Fialho & Schall 1995). Each warm season between 1978 and 2005, fence lizards have been collected from sites scattered over the property, and blood smears made for microscopic examination. Since 1996, dried blood samples have been stored frozen for genetic studies.

Prevalence of the parasite (percent of lizards infected) differs among local sites, with high- and low-prevalence sites often nearby. This pattern has remained for many years (Schall & Marghoob 1995; Eisen & Wright 2001; and J. Seha, subsequent unpublished data). We chose nine sites that maintained the highest relative prevalence over the 28-year study period, and five sites that consistently had the lowest prevalence over time (of course, excluding sites where malaria was always absent). The high-prevalence sites (each 1–3 ha) were within 1.5 km of one another. The low-prevalence sites (3–10 ha) were more scattered at

higher elevations and were within 3.2-km distance of one another. High- and low-prevalence sites are 1.2–4.4 km distant from one another. All sites were within 4.4 km of 39°00′22″N, 123°05′13″W. Mark–recapture studies demonstrate lizards remain in a very local area for years (perhaps their entire adult lives) (Bromwich & Schall 1986; Eisen 2000). Prevalence also varied over the 28-year period. Approximately 20 000 lizards were sampled over the nearly three decades to document such changes in prevalence. During high-prevalence years (1978–1999) at the high-prevalence sites, 25–32% of lizards were infected (combining all sites); this prevalence dropped in later years (2001–2005) to 6–12% (combining all sites). (No sample was taken in 2000.) During years with higher prevalence at the low-prevalence sites, < 1% to 2% of lizards were infected, with prevalence dropping in the low-prevalence years (< 1%).

Here, clonal diversity is compared for several samples: (i) HiYrs. This sample included infections from the nine high-prevalence sites for a series of years with high prevalence (1996–1998). These years followed a long period (from 1978) when prevalence was highest at the field site (above). (ii) LowYrs. Again, these were infections from the nine high-prevalence sites, but only for a set of years with relatively low prevalence (2001–2004). (iii) LowestYr. Infections are included from the nine high-prevalence sites for the most recent low-prevalence year (2005). If clonal diversity is winnowed gradually when transmission is reduced, the effect may not be apparent for several years, so infections for 2005 were assessed separately. Also, 2005 experienced the lowest prevalence (6%) observed over the entire study. (iv) LowSites. This sample included infections surveyed from the five sites that had chronically very low prevalence (~1%). Only infections from the years with higher overall prevalence (1996–1998) are included. Despite substantial collecting effort, the number of infections surveyed is smaller for this sample. Unfortunately, no sample could be included for the low-prevalence sites during the period of years when overall prevalence dropped because infections were extremely rare during that period. No individual lizards were included in more than one sample.

Molecular methods

For assessing overall clonal diversity, and to compare diversity measures for the high-prevalence and low-prevalence times and sites, the number of clones per infection was assayed using three microsatellite markers, Pmx306, 732, and 747 (Schall & Vardo 2007). To detect any distortion in genetic diversity after the possible bottleneck, an additional three loci were scored for the LowYrs and LowestYr samples, Pmx328, 710, and 839. All the loci contain a three-base repeat (ATT). DNA from dried lizard blood was extracted using the DNeasy kit (QIAGEN), and the microsatellite loci amplified by polymerase chain reaction (PCR) using one primer labelled with a fluorescent marker. All primers and PCR conditions are given in Schall & Vardo (2007). PCR product was run on an ABI Prism 3100 Avar Genetic Analyser and the resulting pherograms examined using GENEMAPPER version 3.7 (2004, ABI). Each peak on the resulting pherogram represents a single clone of haploid parasites (length allele).

Infections were chosen from lizards collected from the high-prevalence sites during high-prevalence years of 1996, 1997, and 1998 (the final years of this high-prevalence period for which we have dried blood samples), and from the following low-prevalence years of 2001, 2002, 2003, 2004 and 2005. Thus, the first set of infections were collected after a long period of relatively higher prevalence, whereas the samples from the lower-prevalence years were from 1 to 6 years after the proportion of lizards infected dropped. Infections from chronically low-prevalence sites were sampled only during 1996–1998.

Measures of clonal diversity

Several measures of clonal diversity were calculated. In diverse infections, or infections with low density of one or more clones in the blood, some peaks on the pherograms may be too low to be scored. In preliminary trials, we mixed DNA from single-clone infections that produced similar peak heights on the pherograms, and found that if a clone with a larger allele size (longer repeat) was < 10% the density of another clone, it would often be missed on the pherogram. Thus, low-level infections of a clone could be missed, and overall genetic diversity could be biased low. However, up to six clones were detected when the DNA was mixed in approximately equal proportions prior to PCR. The problem of missing clones in low density is present for any study of clonal diversity in malaria infections, so we used the methods originally used by Anderson *et al*. (2000a), and followed in subsequent studies (Ferreira *et al*. 2002; Bruce *et al*. 2007). These methods also allow comparisons of results for *P. mexicanum* with previous studies on human malaria. To determine allelic diversity for each locus, two criteria were used when reading pherograms resulting from the GENEMAPPER software: highest peak (strongest signal), and all peaks 1/3 the height of the highest peak. For comparisons of clonal diversity within individual infections, all peaks were scored that were above 50 fluorescent units on the pherogram. Peaks were scored only if they demonstrated a standard shape (stutter pattern). Because the parasite is haploid in the lizard blood, each peak represents a clone for that locus. The total possible clones for each infection would be the product of the total for each locus, but this value must greatly overestimate the true clonal diversity. Therefore, we report

clonal diversity within an infection as number of clones for the largest number of clones seen for any locus.

A population-wide measure of overall genetic diversity in the parasite metapopulation among infections is given using several metrics. The total number of alleles and the maximum difference in length are reported for each locus. We also report a genetic diversity metric as $H = (n/n-1)(1 - \Sigma\, p_i^2)$ where n is the number of infections and p_i is the frequency of the ith allele the expected heterozygosity of Anderson *et al*. (2000a). The 95% confidence intervals for H (based on 10 000 replicate H values) were constructed using a bootstrap method described by Dixon (1993). Using the estimate of mutation rates for microsatellites obtained for *Plasmodium falciparum* (1.59×10^{-4}) (Su *et al*. 1999), we calculated the effective population size assuming a stepwise mutation model: $N_e\mu = 1/8\{[1/(1-H)]^2 - 1\}$ (Anderson *et al*. 2000a). During a population bottleneck, rare alleles will be lost before genetic diversity is reduced, resulting in a transient excess in diversity over what is expected under a mutation–drift equilibrium (Nei *et al*. 1975). To determine if such a distortion in genetic diversity measures occurred after the drop in prevalence, we used the method of Cornuet & Lukart (1996) and the BOTTLENECK program (Piry *et al*. 1999).

Table 1 Number of clones (genotypes) of the malaria parasite, *Plasmodium mexicanum*, within individual infections of the vertebrate host, the western fence lizard *Sceloporus occidentalis*. Total clones is estimated as the maximum number of alleles detected for three microsatellite loci. Given is the number of infections, mean number of clones per infection, and percent of infections that were multiclonal. Four samples are compared, samples for a continuous period of years during which prevalence of the parasite was high (HiYrs), subsequent years when prevalence was low (LowYrs), and the most recent low-prevalence year when prevalence was at its 28 years low (LowestYr). The last sample was from a set of sites where prevalence has been very low for the past 28 years (LowSites)

N Clones	HiYrs	LowYrs	LowestYr	LowSites
1	12	11	9	20
2	42	29	24	11
3	37	21	8	6
4	9	3	0	2
5	1	0	0	1
Mean Clones	2.45	2.25	1.98	1.80
N	101	64	41	40
% Multiclonal	88.1	82.8	78.0	50.0

Results

Clonal diversity within infections

Multi-clone infections were common for all three loci (Table 1). Combining all loci and samples, 50–88% of infections contained more than one clone among the samples. Comparisons of clone numbers per infection and numbers of multiclone infections reveal a significant difference among samples ($\chi^2 = 37.8$, $P = 0.0002$, d.f. = 12 for number of clones and $\chi^2 = 25.8$, $P < 0.0001$, d.f. = 3 for number of multiclone infections), but a post-hoc cell contributions test demonstrated this effect was driven only by the relatively low diversity of infections at the low-prevalence sites ($P < 0.05$ for only that cell). That is, whereas 78–88% of infections were multiclonal for the high-prevalence sites for all samples, only 50% of infections were multiclonal for the chronically low-prevalence sites (Table 1; Fig. 1).

Overall clonal diversity

Table 2 presents measures of clonal diversity among infections. Despite repeated efforts, some samples proved refractory to amplification for some loci. Comparing number of alleles per locus, size range (repeat number), and expected heterozygosity for the two methods of scoring alleles (highest peak only vs. all peaks 1/3 height of highest peak on the pherogram) shows either a slightly lower estimate of diversity when scoring only the highest peak, or a very similar result.

After the prevalence of *Plasmodium mexicanum* dropped from the HiYrs to the LowYrs and LowestYr samples, the number of alleles scored in the samples declined. For example, at one locus (Pmx 732), more than half of the alleles present during the high-prevalence years were absent by 2005. Also, fewer alleles were found at the low-prevalence sites. For all loci and all time/site comparisons, 30 alleles were absent after the reduction in prevalence, half were alleles at an original frequency of 1% or less, and 90% were at a frequency of 5% or less in the parasite metapopulation among all infections (Table 3). Only one common allele was absent after the decline in prevalence, one of the Pmx 732 alleles that was 11% frequency during the HiYrs, was absent in the sample by the LowYrs. The loss of scored alleles from high-prevalence to low-prevalence years may reflect a real reduction in frequency or even loss of those alleles in the parasite metapopulation during a genetic bottleneck. Alternatively, rare alleles may simply not be sampled even if they did not change in their frequency. The probability of an allele of frequency of p being missed, by chance, in a sample of n is $(1-p)^n$. Of the 30 alleles possibly lost in the parasite metapopulation, 20 had $a > 5\%$ probability of being missed in the later sampling (range 6% to 26%) if their frequency remained the same as during the high-prevalence years. However, picking the 11 alleles with the highest probability of being missed (> 25%), the probability of missing all is 3.2×10^{-7} (product of the 11 probability values). It is therefore highly likely that some of

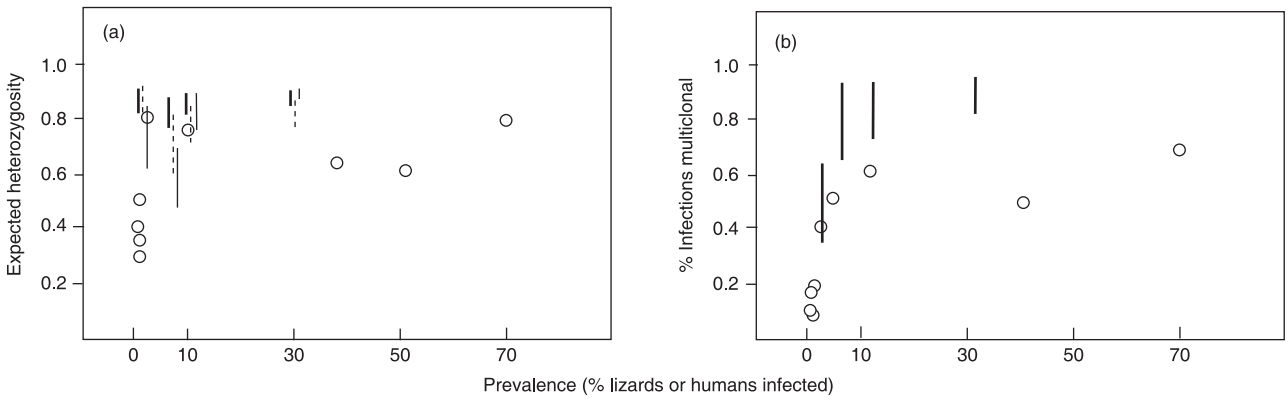

Fig. 1 Measures of clonal diversity for *Plasmodium mexicanum* compared with those for *Plasmodium falciparum* (*P. falciparum* data extracted from Anderson *et al*. 2000a). For both panels, four samples are compared for *P. mexicanum* that differed by prevalence. These are (from lowest to highest prevalence) LowSites, LowestYr, LowYrs, and HiYrs (see Methods). For each panel, confidence intervals for the *P. falciparum* data could not be calculated because only summary measures were available from the literature. (a) A measure of overall clonal diversity (expected heterozygosity, *H*) for *P. falciparum* and *P. mexicanum*. Data for *P. falciparum* are indicated with open points. Results for *P. mexicanum* are given as 95% confidence intervals for *H* to allow comparisons among loci, samples, and with the *P. falciparum* data. Confidence intervals are given for three loci for *P. mexicanum* (Pmx 306 heavy line, 732 dashed line, 747 light line, from left to right for each sample). (b) Similar data for percent of infections that were multiclonal, with 95% confidence intervals for percents given in Table 1.

Table 2 Measures of clonal diversity for the malaria parasite *Plasmodium mexicanum* in its lizard host based on alleles for six microsatellite loci. Given are results for sites with relatively high prevalence of the parasite in lizard hosts at the study area for years with overall high (HiYrs) and low (LowYrs) prevalence, the last year with low prevalence (LowestYr), and for sites during the high-prevalence years which consistently show low prevalence of the parasite (LowSites). Clonal diversity measures are number of alleles detected for each locus (# Alleles), range in number of a three base repeat for the microsatellite for each locus (Size range in number of repeats), expected heterozygosity (*H*), effective population size (N_e), and proportion of the infections that were multiclonal (Multiclonal). Two measures are given, using all peaks on the pherograms that were > 1/3 the height of the highest peak (first value) and only using the highest peak (second value). For the estimate of percentage of infections that were multiclonal, all peaks > 50 fluorescent units on the pherograms were counted. Sample sizes are given in the first column as (total number of infections surveyed for that locus, total number of alleles counted among all infections)

Locus	#Alleles	Size range	*H*	$N_e \times 1000$	% Multiclonal
Pmx306					
HiYrs (92, 131)	17 (17)	19 (19)	0.89 (0.86)	64.2 (39.3)	59.1
LowYrs (62, 88)	15 (14)	18 (18)	0.88 (0.87)	52.1 (49.2)	48.4
LowestYr (40, 51)	10 (9)	15 (15)	0.85 (0.85)	34.1 (34.1)	45.0
LowSites (31, 46)	14 (12)	13 (13)	0.89 (0.88)	64.2 (53.8)	45.0
Pmx732					
HiYrs (70, 89)	17 (14)	33 (32)	0.83 (0.78)	26.4 (15.4)	33.0
LowYrs (64, 88)	12 (11)	33 (33)	0.81 (0.76)	21.4 (12.7)	50.0
LowestYr (38, 45)	7 (7)	26 (26)	0.82 (0.70)	23.5 (7.9)	39.5
LowSites (24, 25)	11 (10)	22 (22)	0.92 (0.91)	122.0 (96.3)	12.5
Pmx747					
HiYrs (98, 155)	16 (14)	21 (21)	0.90 (0.89)	77.8 (64.2)	67.3
LowYrs (64, 76)	15 (13)	17 (17)	0.88 (0.85)	40.3 (34.2)	34.4
LowestYr (40, 61)	9 (7)	13 (13)	0.64 (0.51)	5.3 (2.5)	70.0
LowSites (34, 39)	9 (9)	18 (18)	0.78 (0.78)	15.4 (15.4)	32.0
Pmx328					
LowYrs (68, 79)	4 (3)	11 (10)	0.41 (0.38)	1.5 (1.2)	14.0
LowestYr (44, 45)	3 (3)	10 (10)	0.24 (0.24)	0.6 (0.6)	2.0
Pmx710					
LowYrs (63, 77)	15 (14)	22 (22)	0.88 (0.94)	217.5 (53.8)	21.0
LowestYr (23, 26)	8 (8)	11 (11)	0.87 (0.86)	42.9 (38.7)	13.0
Pmx839					
LowYrs (69, 87)	13 (13)	36 (36)	0.87 (0.87)	45.7 (45.7)	25.0
LowestYr (33, 39)	8 (8)	13 (13)	0.85 (0.85)	34.6 (33.3)	18.0

Table 3 Number of alleles that were present in infections of *Plasmodium mexicanum* in its lizard host during a period of years with high overall prevalence (25–32%, 1996–1998), but were missing in samples taken after prevalence dropped (6–12%, 2001–2005). Three loci are given with the initial frequency of the lost clones. Note that most alleles missing in later samples were initially rare in the parasite metapopulation

	Frequency of alleles in 1996–1998 sample					
Loci	< 1–1%	2%	3%	4%	5%	6–11%
Pmx 306	3	5	1			
Pmx 732	5	1	2		1	3
Pmx 747	6		1	1	1	

Table 4 Number of microsatellite loci (total of six scored) revealing an excess in expected heterozygosity for *Plasmodium mexicanum* when compared to expected values under mutation–drift equilibrium (calculated with BOTTLENECK software). Two mutation models were employed, the infinite alleles model (IAM) in which any size allele can mutate to any other size, and the stepwise mutation model (SMM) in which any size allele can mutate only to an increase or decrease of one repeat unit. Two samples of genetic diversity of *P. mexicanum* are given. The first (LowYrs) was a period of 4 years of low infection prevalence that followed a long period of much higher prevalence, and the second (LowestYr) was the next year which had the lowest prevalence recorded over a 28-year period. Significance level is given under the Wilcoxon test for number of loci under expectation of an excess in expected heterozygosity

	IAM		SMM	
Sample	*N* loci *H* excess	*P*	*N* loci *H* excess	*P*
LowYrs	5	0.016	0	1.000
LowestYr	5	0.039	2	0.945

the alleles, perhaps most, were actually lost in the parasite metapopulation. Also, no new alleles were detected in the low-prevalence samples.

The reduction in number of alleles scored after prevalence declined was not associated with a reduction in overall diversity (*H*). Figure 1 shows substantial overlap of confidence intervals for *H* estimated for the three loci for all samples.

Theory and simulations reveal that a substantial population bottleneck will result in a short-term increase in genetic diversity compared to that expected under a mutation–drift equilibrium based on the number of alleles present (Nei *et al*. 1975; Cornuet & Luikart 1996). A minimum of six loci are required to test for this distortion after a possible bottleneck. Comparing observed genetic diversity (expected heterozygosity based on actual clonal frequencies) and heterozygosity at equilibrium for the number of alleles (BOTTLENECK program), we found a significant excess in expected heterozygosity under the infinite alleles model of mutation (IAM) in which any length allele can mutate to any other length, but no evidence of an excess under the stepwise mutation model (SMM) in which any length allele can mutate only to an allele one repeat longer or shorter (Table 4).

Discussion

In contrast to the situation for human malaria parasites, the genetic structure of *Plasmodium mexicanum* has not been disturbed by substantial population mixing over broad geographical areas, human intervention to reduce transmission, or selective sweeps initiated by drug treatment. Thus, the clonal diversity patterns of *P. mexicanum* should be a product strictly of events during the parasite's life cycle and changes in transmission intensity and prevalence driven by environmental or endogenous factors. This parasite system therefore provides a clear test of the view that malaria parasites should have reduced diversity because of loss of clones during transmission, loss of diversity in areas with low transmission intensity, or bottlenecks during periodic low transmission periods.

We scored clonal diversity for *P. mexicanum* at three microsatellite loci over a 3-year period ending a much longer period with high prevalence (from 25 to 32% of lizards infected from 1978 to 1998). Prevalence subsequently dropped from 2001 to 2005, and we scored clonal diversity for 2001–2004, and then 2005, the year with lowest prevalence (6%). These samples were all taken at sites where prevalence of the parasite has been highest at the study region. A set of other sites, that maintained very low prevalence (1% or lower) over the years was also sampled. The results reveal that *P. mexicanum* maintained substantial clonal diversity for all time periods and sites sampled. Overall genetic diversity (*H*) was high for all samples, including the low-prevalence sites where genetic diversity was not significantly different than for sites with much higher prevalence (Fig. 1). The measures of overall clonal diversity did not differ significantly among samples (Fig. 1). The single measure of clonal diversity that declined from high- to low-prevalence years was the number of alleles scored in the samples. Although these 'lost' alleles could have remained at their initially low frequency (or even increased) and simply were missed by the sampling program, it is more likely that these alleles either dropped much lower than their original frequency or were lost in the parasite metapopulation. During population bottlenecks, rare alleles are most likely to be lost first (Nei *et al*. 1975; Luikart *et al*. 1998), and most of the alleles not detected in later years had been rare alleles earlier.

Most striking in these comparisons is the high proportion of infections that were multiclonal, even for the last year sampled (2005) after 4 years of low prevalence. At the sites with chronically very low prevalence, there was a significantly lower proportion of multiclonal infections, but still half of the infections contained more than one clone. These results, and those for human malaria parasites (below), argue that mixed-clone infections are generally common for *Plasmodium* such that two to several genotypes of parasite will often co-exist in the same vertebrate host. These common genetically complex infections could lead to selection for competitive interactions among clones, or perhaps cooperation. Also, mixed-clone infections may be more harmful to the host (Read & Taylor 2001) so the observed genetic structure should play an important role in the evolution of virulence.

Changes in prevalence over time, and variation over space, is common for malaria parasites of both human and nonhuman vertebrate hosts (review in Schall & Marghoob 1995). However, such variation may not be sufficient to reduce genetic diversity; that is, a reduction in prevalence from 30% to 6% or even to 1% may not actually create a genetic bottleneck if the absolute number of hosts infected remains high. During a genetic bottleneck, as rare alleles are lost, the observed genetic diversity will appear higher than expected under a specific model of mutation–drift equilibrium (Nei *et al*. 1975; Luikart *et al*. 1998). This excess in heterozygosity can thus test for any actual genetic bottleneck. Contradictory results emerged for two mutation models for *P. mexicanum*. Under the IAM, a significant excess in heterozygosity was observed, even scoring only six loci, but no such excess was observed under the SMM (Table 4). A similar result was found for the human malaria parasite, *Plasmodium falciparum*, for sites where a bottleneck was suspected (Machado *et al*. 2004). Simulations demonstrate that the predicted heterozygosity excess is difficult to detect under SMM, but becomes obvious when there is even a slight tendency for alleles to mutate via the IAM model (Luikart *et al*. 1998). Thus, the results are equivocal for the *P. mexicanum* system, leaving an open question as to the real impact of reductions in prevalence for malaria parasites. In any case, genetic diversity remained high for the parasite metapopulation, and number of mixed infections were common, even for sites where prevalence has been low for many years.

Our methods in scoring clonal diversity followed those of Anderson *et al*. (2000a) so that we can compare the results for *P. mexicanum* with a worldwide survey of genetic diversity in *P. falciparum* in humans (Fig. 1). The relationship between transmission intensity (estimated using prevalence) and both proportion of infections that were multiclonal and the overall clonal diversity (H) for *P. falciparum* are nonlinear. Some areas with low transmission revealed low clonal diversity and fewer mixed infections, but other areas with similar transmission maintained high clonal diversity (Fig. 1). For *P. mexicanum*, overall clonal diversity was high for all levels of prevalence, and mixed infections were less common only for the lowest prevalence sample (LowestYr = 2005).

The similarity of the results for a lizard malaria parasite and *P. falciparum* worldwide suggests that high clonal diversity of malaria parasites may be a general situation. This conflicts with Tibayrenc *et al*. (1991) who suggest that malaria parasites may maintain low levels of genetic diversity, even uniclonal at some sites (see Durand *et al*. 2003 for a similar result using microsatellite markers). Several features of *Plasmodium* biology would seem to favour a reduction in genetic diversity. Assortative mating within the vector between identical gametes would yield identical haploid cells for transmission to the next vertebrate host, although the degree of mating between identical gametes remains an open question for *Plasmodium* (Anderson *et al*. 2000b; Razakandrainibe *et al*. 2005). Clones could be lost during transmission between vertebrate and insect hosts, thus gradually reducing the genetic diversity in the metapopulation. Large scale bottlenecks could occur during periods of low transmission when effective population size declines (Price 1980). Despite the plausibility of these arguments, measures of genetic diversity for human malaria parasites using surface proteins revealed multiclonal infections are common, especially in areas with high transmission (Paul *et al*. 1995; Gilbert *et al*. 1998; Paul *et al*. 1998; Babiker *et al*. 1999; Felger *et al*. 1999; Konaté *et al*. 1999; Cui *et al*. 2003). Use of surface protein genes to probe the clonal diversity of *Plasmodium* infections may be questioned because of the very high mutation rate for the loci and selection for diversity to match the host immune challenge (Hughes & Hughes 1995; Anderson *et al*. 2000a; Mascorro *et al*. 2005). Microsatellite loci have been proposed to be better indicators of clonal diversity; studies using these markers also reveal substantial clonal diversity within and among infections for two species of human malaria parasite (Anderson *et al*. 2000a; Ferreira *et al*. 2002; Durand *et al*. 2003; Bruce *et al*. 2007). We chose to use microsatellites in the study of *P. mexicanum* because of their more likely neutral nature (and surface protein genes have not been discovered for this parasite species).

The results of studies on human malaria parasites as well as our findings for *P. mexicanum* suggest an intriguing question. Why does overall genetic diversity remain high and multiclone infections remain common even during long periods of reduced transmission and at sites with low parasite prevalence? At the Hopland sites with only 1% or less of lizards infected over a period of decades, an estimate of overall clonal diversity (expected heterozygosity) is as high as 0.91, and fully half of infections are multiclonal. Likewise, genetic diversity remains high in metapopulations of *P. falciparum* at some sites with low prevalence.

Gemmill *et al*. (1997) proposed that sexual reproduction in parasites functions to generate genetic diversity in offspring to face a changing environment as hosts develop resistance to infection. The results for *Plasmodium* suggest there may be selection favouring genetic diversity in the parasite, perhaps as a result of the diversity of immune responses developed by hosts.

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