Microsatellite loci over a thirty-three year period for a malaria parasite (*Plasmodium mexicanum*): bottleneck in effective population size and effect on allele frequencies

J. J. SCHALL* *and* K. M. ST. DENIS†

Department of Biology, University of Vermont, Burlington, VT 05405, USA

(*Received 21 February 2012; revised 12 April, 16 May and 12 June 2012; accepted 13 June 2012; first published online 5 September 2012*)

SUMMARY

Changes in population allele frequencies may be driven by several forces, including selection and drift, and are revealed only by sampling over many generations. Such studies, however, are rare for protist parasites. Microsatellite allele frequencies for 4 loci were followed in a population of *Plasmodium mexicanum*, a malaria parasite of lizards in California USA at 1 site from 1978 to 2010. Rapid turnover of the lizards indicates the parasite was studied for a minimum of 33 transmission cycles and possibly twice that number. Sample sizes ranged from 841 to 956 scored parasite clones per locus. DNA was extracted from frozen dried blood and blood removed from stained blood smears from the earliest years, and a verification study demonstrated DNA from the blood smears provided valid genetic data. Parasite prevalence and effective population size (Ne) dropped after 2000, remaining lower for the next decade. For 2 loci, allele frequencies appeared stable for the first 2 decades of the study, but changed more rapidly after the decline in prevalence. Allele frequencies changed more gradually for the other 2 loci. Genetic drift could account for changes in allele frequencies, especially after the drop in prevalence and Ne, but the force of selection could also have driven the observed patterns.

Key words: microsatellites, allele frequency change, long-term genetic studies, *Plasmodium mexicanum*, malaria parasite.

INTRODUCTION

Microparasites have the potential for rapid evolutionary change because of their short generation time (relative to their hosts), population substructure among individual hosts, intense co-evolutionary selective pressures, and sudden shifts in effective population size when parasite prevalence waxes and wanes (Price, 1980; May, 1991). For example, models of co-evolution for parasites with such life histories predict genetic polymorphisms that will cycle or even follow chaotic patterns over short time-scales (May, 1991). Genetic drift likewise should have a potent influence because parasites exist in non-equilibrium population sizes both within individual hosts over the course of an infection as well as when prevalence among hosts changes with shifting environmental conditions (Price, 1980). Evidence for the predicted rapid evolutionary change requires long-term data on allele frequencies appropriate for the life cycle of each parasite. Such data are available primarily for viruses (Ghedin *et al*. 2005), but are still scant for other parasites. An ideal system to examine allele frequency changes over time would be the malaria parasites (*Plasmodium* and related genera, *sensu* Martinsen *et al*. 2008). Studies over 4–5 years reveal shifts in microsatellite allele frequencies for *P. falciparum* (Orjuela-Sanchez *et al*. 2009) and *P. vivax* (Ferreira *et al*. 2007) which argue for rapid evolution of these human malaria parasites, as predicted. We have monitored allele frequency changes at 4 microsatellite loci for *P. mexicanum* in its natural host, the western fence lizard, *Sceloporus occidentalis*, for more than 3 decades to determine whether the frequency of alleles change rapidly. The prevalence of *P. mexicanum* at the study site dropped suddenly approximately 2 decades into the study, and thus opens the possibility of a bottleneck in effective population size and resulting genetic drift.

Microsatellite genetic loci offer a powerful tool for studies on the ecology and evolution of malaria parasites. These short tandem repeats occur every 1000 nt in the genome of *P. falciparum* and have also been found in several other *Plasmodium* species (Anderson *et al*. 2000; Schall and Vardo, 2007; Havryliuk *et al*. 2008). Microsatellite length alleles are assumed to be neutral (Selkoe and Toonen, 2006). For malaria parasites, microsatellite loci have been used to determine overall genetic diversity related to transmission intensity (Anderson *et al*. 2000; Vardo and Schall, 2007), geographical genetic differentiation (Conway *et al*. 2001; Abdel-Muhsin *et al*. 2003; Zhong *et al*. 2007; Fricke *et al*. 2010), and when linked to loci under selection, to follow selective sweeps after introduction of drug therapy (Nair *et al*.

* Corresponding author: Tel: +802 656 0448. Fax: +802 656 2914. E-mail: jschall@uvm.edu
† Current address: Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104-6087, USA.

Parasitology (2013), **140**, 21–28. © Cambridge University Press 2012
doi:10.1017/S0031182012001217

2003; Bonizzoni *et al*. 2009). However, long-term studies on microsatellite allele frequencies for any malaria parasite are lacking. Such studies have proven highly useful for many free-living organisms, such as revealing the changes in effective population size after a bottleneck in density (Hutchinson *et al*. 2003; Johnson *et al*. 2004). Similar data should open windows into events that shape the genetic structure of malaria parasites.

We have quantified allele frequencies for 4 microsatellite loci in the genome of *P. mexicanum* at a single study site over a 33-year period from 1978 to 2010. The typical 1-year lifespan of the lizard hosts means the study covered a minimum of 33 transmission cycles, and data on the ecology of the system suggest there could have been twice that number of cycles (Schall, 1996). Our method was to use frozen blood samples stored from 1996 to 2010, and blood removed from Giemsa-stained blood smears for earlier years. Use of the blood smears as a source of DNA for molecular studies was validated. Fifteen of the annual samples were examined for the overall study period. *P. mexicanum* at the site has not been under any anthropogenic selective pressures, and previous studies have shown local genetic differentiation over only several km distance (Fricke *et al*. 2010) showing that migration should have minimal influence. Prevalence of the parasite has been monitored during most years, and showed a sudden drop around 2000 (Vardo and Schall, 2008). Our goal was to determine if allele frequencies change rapidly over time as predicted by both coevolution and drift models of evolution of microparasites (Price, 1980; May, 1991).

MATERIALS AND METHODS

Collection of samples

All infected lizards used for this study were collected at the University of California Hopland Research and Extension Center (HREC) near the town of Hopland in Mendocino County, California, where the parasite-host system has been under study since 1978 (Schall, 1996; Fricke *et al*. 2010; Neal and Schall, 2010). The vertebrate host is the western fence lizard *Sceloporus occidentalis*, and insect vectors are 2 species of sand flies, *Lutzomyia vexator* and *L. stewarti*. Lizards were sampled each year by noosing, and returned to their point-of-capture within 24 h after several drops of blood were taken to produce a thin blood smear for staining (Giemsa stain), and to store dried and frozen blood on filter paper. Dried blood dots were available for 11 of 15 years from 1996 to 2010. Earlier samples were obtained by removing part of the stained blood smear from microscope slides stored at room temperature (below). All samples were collected from sites within 1·5 km of 39.0044N, 123.0863W; previously, no differentiation in the parasite within this area was detected (Fricke *et al*. 2010). No lizard was sampled in more than 1 year, primarily because 1 year is the typical life span of the lizards (Eisen, 2001).

Parasite prevalence

Lizards have been sampled to determine infection status for most summers of the overall study period from 1978 to 2010 (29/33 years). Stained blood smears are scanned until ~10 000 erythrocytes have been examined to score the lizard as infected or not infected. A highly sensitive PCR-based method to detect very weak infections reveals that very few infections are missed by microscopic examination of the smears (Perkins *et al*. 1998, and subsequent unpublished studies). Prevalence is given as percentage of lizards infected.

Extracting DNA and genotyping parasites

DNA was extracted from stored blood dots taken during 1996–1998, 2002–2003, 2005–2010 (11 samples), and from the stained slides for 1978–1979 and 1986–1987 (4 samples). DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA) and the provided protocol (Vardo and Schall, 2007). To reduce the possibility of cross-contamination, polymerase chain reaction (PCR) runs were done in a UV-PCR box, with all surfaces frequently exposed to UV light, and the air HEPA filtered. When samples from stained slides were processed, DNA was also extracted under the box and dedicated extraction reagents and a new dedicated set of pipettes were used.

Parasites were genotyped for 4 microsatellite loci, Pmx306, Pmx747, Pmx732, and Pmx839, using PCR conditions and primers reported by Schall and Vardo (2007). Flanking regions for these loci were compared with published (GENBANK) genomes of other malaria parasites, without any significant homology. For each reaction, 1 Ready-to-Go PCR bead (GE Healthcare, Piscataway, NJ) containing DNA polymerase, dNTPs, and buffers was combined in a 25 μl reaction mixture containing 19 μl of water, 1 μl of each of 10 μM forward and reverse primers, and 4 μl of the extracted DNA. A negative control was included with each batch of samples processed. PCR product was visualized on a 1% agarose gel, and the density of the band was used to determine the dilution of each sample for genotyping. The diluted PCR product (1 μl) was added to 15 μl of a mixture of Hi-Di formamide (ABI, Foster City, CA, USA) and LIZ500 size standard (ABI). Samples were run through an ABI 3730xl Genetic Analyzer instrument (Cornell University Core Lab), and data generated by the instrument were analysed using GeneMapper 3.5 software.

Because malaria parasites are haploid in the vertebrate host's blood, each peak on the resulting pherogram was considered a single clone (individual) of parasite present in the infection. Thus, if 3 peaks appeared on the pherogram for a locus, each was counted as an individual, resulting in sample sizes greater than the number of infected lizards sampled. Two major sources of error could result in either random error or biased estimates of allele frequencies (Hastings *et al*. 2010). First, the PCR could amplify some alleles more efficiently than others and/or a clone in low density in the blood may be missed because of the competitive nature of the amplification. Second, multiple clones could have the same microsatellite allele, and in mixed infections the allele would be undercounted. The most common clone would more likely to be confounded in this way, and when mixed-clone infections are more common (times or places with higher infection prevalence) the frequency of the common clone would be underestimated. However, previous studies of this malaria parasite using simulated infections (by mixing extracted DNA from single-clone infections in various proportions), experimentally induced infections, and natural infections followed over weeks to years demonstrated no significant errors in detecting alleles, nor bias in counting any particular allele (Ford *et al*. 2010; Ford and Schall, 2011; Vardo and Schall, 2007; Vardo-Zalik and Schall, 2009; Vardo-Zalik *et al*. 2009). A previous study (Vardo and Schall, 2007) found that mixed-clone infections were not more common prior to the drop in prevalence. Hastings *et al*. (2010) found that only major changes in prevalence produced potential errors in counting alleles. Counting only the most prominent peak on the pherograms also may reduce chances of error (Anderson *et al*. 2000), but in the previous study on *P. mexicanum* (Vardo and Schall, 2007), estimates of mixed-clone infections and genetic diversity did not differ when only the tallest peak on electropherograms, the tallest plus any 1/3 as tall, and all peaks were used in analysis. In this study all the peaks were used to increase sample size, and thus statistical power. However, these potential sources of error still must be considered when interpreting the results (below).

Validating samples from stained smears

To verify the value of using DNA extracted from stained blood smears, infections were genotyped in 2010 by scratching stained blood from smears made in 1996–1998, the earliest date for which dried blood was also available, and comparing results with those from DNA extracted from blood dots. Four years earlier, the dried blood samples were used to genotype infections, so this greatly reduced the likelihood that there was contamination between the controls (dried and frozen blood) and the trials (blood from blood smears).

Statistical analysis

Comparisons of microsatellite allele frequencies were performed using the Shannon diversity metric based on information theory available on the GenAlEx program (Peakall and Smouse, 2006). This index has been widely used in studies of diversity, including ecological communities, and genetic variation in humans and viruses (Sherwin *et al*. 2006). The method allows use of haploid data and has statistical properties ideal for comparison of microsatellite allele frequencies between years. Sherwin *et al*. (2006) and Rossetto *et al*. (2008) use simulations and empirical results to validate the Shannon metric in detecting differences between samples. Other analysis used JMP 8 (SAS, Cary, NC).

Estimation of the effective population size (Ne), or the breeding population, is notably challenging (Wang, 2005), and many methods have been proposed. Two are used here. The first is the method of Anderson *et al*. (2000). This measure of Ne uses the genetic diversity in the population and an estimate of microsatellite mutation rates for *Plasmodium*. Although an error in the estimated mutation rate would alter the value of Ne calculated, changes in Ne would still be detected correctly because mutation rate is entered as a constant. The second method of Garza and Williamson (2001) uses the range in microsatellite allele sizes. During a bottleneck, rare alleles are likely to be lost, thus reducing the number of alleles detected. The range in allele sizes (number of repeat units) would also decline, but only if the lost allele was at either end of the size range. Thus, the allele number/allele size range ratio should drop after a bottleneck. Their metric is M, the ratio of number of alleles : range in number of repeats for each locus. Garza and Williamson (2001) used both simulations and data from actual populations that were known to have experienced a drop in demographic population size to validate the method. That is, the value of M dropped suddenly after both the simulated drop in Ne and drop in demographic population size for real populations. The method of Garza and Williamson thus has the advantage of having been empirically tested against real data for declining populations. Calculation of M assumes a step-wise mutation model in which microsatellite alleles mutate by shifting 1 repeat unit (in our study, 3 bases) rather than additions or deletions of blocks of repeat units. The allele sizes seen in our data (see Results section) support the step-wise mutation model. Use of this method requires sample sizes greater than twice the number of alleles, and this held for our analysis.

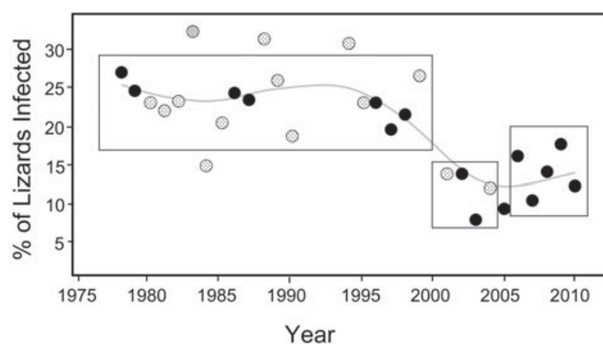

Fig. 1. Prevalence of *Plasmodium mexicanum* in its vertebrate host, the western fence lizard *Sceloporus occidentalis* at a study region in Northern California over 33 warm seasons. Lizards were sampled with replacement every year using identical methods at the same sites. Consecutive years with prevalence not significantly different (χ^2 tests) are enclosed with boxes. A sudden drop in parasite prevalence occurred prior to the 2002 sample. A polynomial fit (curve) to the data is shown. Dark circles are those for which data are available for allele frequencies of 4 microsatellite loci.

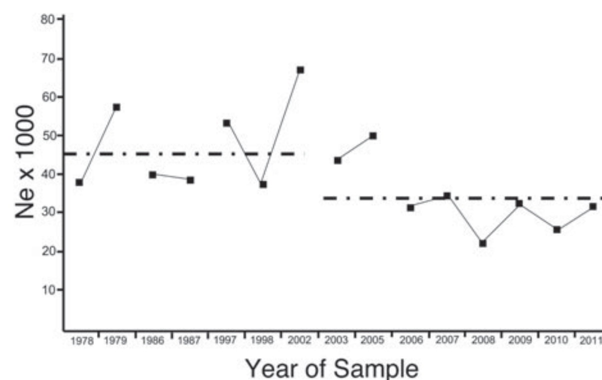

Fig. 2. Estimate of effective population size (Ne) for the lizard malaria parasite *Plasmodium mexicanum* at a study site in northern California for sampled years from 1998 to 2010, calculated based on genetic diversity and an estimate of microsatellite mutation rates (see text). Each point is the mean for Ne estimated from 4 microsatellite loci. Points for sequential years are connected. Means for years before a drop in prevalence of *P. mexicanum* in its lizard host and after the drop are indicated with the vertical dashed/dotted lines.

RESULTS

Validation for DNA extracted from stained smears

We compared results for 28 samples from infected lizards collected from 1996 ($N = 18$) 1997 ($N = 2$), and 1998 ($N = 8$) for 1 to 4 microsatellite loci from dried/frozen blood and from the scratched-off stained blood smears. A total of 49 pairs of pherograms could be compared for frozen DNA and from the scratched blood smears among the 4 loci, with 47 showing the same alleles. One sample (for Pmx747) showed the same allele for the smear *vs* frozen blood, but also an extra allele for the blood smear, and a second sample (for Pmx732) showed a different allele for the blood smear and frozen blood (3 repeat units different). Six of the samples from blood smears did not yield pherogram peaks for any locus, indicating severely degraded DNA that had been subjected to absolute methanol and staining, and 6 trials produced unreadable results for 1 or more loci. These unreadable results were either no signal, or clearly spurious results with many peaks across a range of fragment lengths. In summary, of readable pherograms, 96% produced the same results for the frozen blood *vs* blood cells taken from the stained blood smears that were up to 15 years old and stored at room temperature in the laboratory. For the overall study, any pherogram that showed a very weak signal, multiple peaks, or no characteristic stutter for the blood smear samples, resulted in that sample being discarded to insure high-quality data.

Parasite prevalence over time

Figure 1 presents data on overall prevalence of *Plasmodium mexicanum* in its fence lizard host over the study period. Prevalence remained stable from 1978 to 1999 at ~17–27% (within the 95% confidence interval for the proportion of lizards infected), but dropped between 2000 and 2001 to ~6–12% until 2005 when prevalence began to rise slowly to about 15% by 2010 and 2011.

Effective population size

A demographic bottleneck (drop in prevalence) about 2000 could have resulted in a drop in effective population size (Ne) at that time. Figure 2 shows the estimated Ne based on the method of Anderson *et al*. (2000) using genetic diversity and an estimate of microsatellite mutation rate for the sampled years. Mean Ne for the years prior to 2000 of about 45 000 for the study sites dropped to 32 000 after 2000. Thus, the drop of about 30% for Ne was lower than the drop in prevalence by about half. The method of Garza and Williamson (2001) found that the ratio of allele number to allele size range (M) dropped for 3 loci (Pmx306 M = 0·895 to 0·667; Pmx732 M = 0·515 to 0·269; Pmx747, M = 0·761 to 0·692), but rose slightly for the locus with the lowest number of alleles (Pmx839 M = 0·333 to 0·409).

Allele frequencies over time

If each peak on a pherogram represented 1 clone of parasite, overall 841–956 clones were sampled for each of the 4 microsatellite loci. Table 1 presents the number of infected lizards sampled, with data on the proportion of those infections that were multiclonal and number of alleles (= haploid parasites) scored for each locus each year. The full data set

Table 1. Number of infected fence lizards sampled to score microsatellite alleles for the malaria parasite *Plasmodium mexicanum*

(Number of infected lizards, proportion of infections carrying more than a single clone (based on having at least 2 alleles at any of 4 microsatellite loci), and number of alleles (clones for a haploid parasite) scored for each of 4 microsatellite loci, Pmx306, Pmx732, Pmx747, and Pmx838.)

Year of sample	*N* Infections	% Mixed-clone	Pmx306	Pmx732	Pmx747	Pmx838
1978	32	50	38	12	28	36
1979	24	79	46	23	56	69
1986	37	51	39	29	43	45
1987	34	38	42	36	43	36
1996	51	80	49	26	61	38
1997	50	88	94	40	81	38
1998	29	72	38	41	48	11
2002	42	76	64	83	53	45
2003	18	83	34	31	25	23
2005	54	91	59	65	60	45
2006	68	91	99	145	85	84
2007	50	88	58	42	42	73
2008	66	67	58	52	55	58
2009	118	64	88	88	106	92
2010	103	47	150	156	138	147

with sample sizes for each year and allele frequencies for all years are available from the authors or at http://www.uvm.edu/~jschall/MicroSatData.html.

Figure 3 summarizes allele frequency changes by combining sequential years with no significant shift in frequency for each microsatellite locus (Shannon Information Metric, with $P=0{\cdot}05$ as the cut-off for significant difference). For 2 loci, Pmx732 and Pmx839, allele frequencies appeared stable for the first 2 decades (1978–1998) of the study, but after the drop in prevalence and Ne, allele frequencies were less stable, even changing significantly over a single year. For the other 2 loci, there appears to have been a more gradual change in allele frequencies, although there were periods of a decade with no significant change. To determine whether there was greater variation in allele frequencies before compared with after the drop in prevalence, all pairs for the first 7 samples and the last 7 samples were compared to count the number of significantly different pairs. For loci Pmx732 and Pmx839 this comparison showed significantly more variation after the drop in prevalence (G tests, $G=13{\cdot}72$, $P<0{\cdot}05$, and $G=13{\cdot}72$, $P<0{\cdot}05$ respectively). For loci Pmx306 and Pmx747, there was no difference in the number of significantly different samples before and after the drop in prevalence (G tests, $G=0{\cdot}653$, $P>0{\cdot}05$ and $G=0{\cdot}024$, $P>0{\cdot}05$ respectively). For 2 loci, Pmx747 and Pmx839, there was no significant difference between the latest year (2010) and the earliest years (1978, 1979), although there were unique alleles found in either the early or late sample (5 for Pmx747 and 4 for Pmx838).

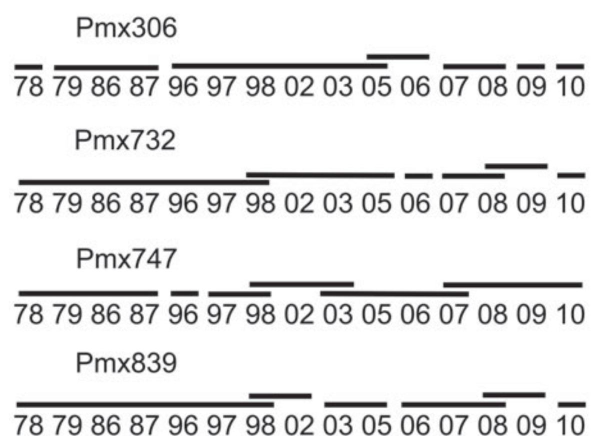

Fig. 3. Allele frequencies compared across years (1979–2010) for 4 microsatellite loci in the genome of *Plasmodium mexicanum*, a malaria parasite of the western fence lizard, *Sceloporus occidentalis*, in northern California. Loci Pmx306, Pmx732, Pmx747, Pmx839 are shown; sequential years with no significant difference in allele frequencies indicated with bars (Shannon information metric, $P>0{\cdot}05$).

DISCUSSION

Duration of study: number of parasite transmission cycles

Microsatellite allele frequencies were determined for a large sample of individual clones for the malaria parasite, *Plasmodium mexicanum*, for 15 years sampled over 33 warm seasons. Each sampled lizard in the long-term study at the site has been individually marked by toe clips and with a mark on the dorsal body surface (example in Bromwich and Schall, 1986). Sampled animals are

frequently seen, and often recaptured, during intensive mark-recapture studies during any single year, but are more rarely taken between 2 years, and almost never between 3 years (Eisen, 2001). The lizard population appears to be turning over every year, and parasites sampled each year most likely represent a new generation. None of the lizards used in this study were recaptures between years. Even if only a single transmission cycle occurs each year in the seasonal environment of northern California, the rapid turn over of the vertebrate host indicates 33 transmission cycles occurred during the study period. If more than a single cycle occurs per year, the number of parasite generations could have been far greater. Thus, the study provides an opportunity to observe allele changes in a malaria parasite over many generations and to observe genetic changes in a malaria parasite that has not been disturbed by public health interventions.

Validation of samples from stained blood smears

DNA was extracted from stored dried and frozen blood samples, and also from blood removed from stained blood smears to extend the study back another decade. A verification study showed nearly identical results from DNA taken from 15-year-old blood smears and frozen dried blood that had been processed several years earlier. Thus, such samples are shown to be useful for long-term studies on the genetics of this malaria parasite, and most likely would be feasible also for other species. However, the observation that samples from the most recent years (2009 and 2010) was similar for 2 loci from the earliest samples suggested the possibility of contamination. We discount this possible source of error for several reasons. First, the blood smears were processed from DNA extraction through PCR under a workstation with filtered positive air pressure and frequent UV 'burning' of the work area, and using a dedicated new set of pipettes. Second, negative controls were included with each batch of samples for DNA extraction and PCR, which never revealed contamination (bands on the agarose gel or peaks on the pherograms). Third, the 2009 samples had been genotyped the year earlier in the laboratory at a different work area. Last, although the earliest versus latest samples did not differ significantly, they revealed different, private alleles in each sample for both loci.

Allele frequencies over time: migration, mutation, drift and selection

The natural history of malaria parasites (and other protists) is likely to lead to rapid changes in allele frequencies driven by migration and mutation, but especially by drift and selection. Changes in microsatellite allele frequencies have been seen over only a period of a few months to years for human malaria parasites (Ferreira *et al*. 2007; Orjuela-Sanchez *et al*. 2009). In contrast, our results showed different patterns among loci. For 2 loci, microsatellite allele frequencies did not differ over the first 2 decades of the study (at least 20 transmission cycles), but showed a significant increase in variability among years later in the study period. The other 2 loci were more variable among years, although not changing as rapidly as reported for the human malaria parasites.

No obvious environmental changes occurred at the site when allele frequencies shifted (such as weather, density of lizards; *unpublished observations*) except for the drop in proportion of lizards infected (parasite prevalence).

Four forces can change allele frequencies in a population: migration, mutation, drift, and selection (Freeman and Herron, 2007). We discount the possibility that either migration (and resulting gene flow) or mutation played a significant role in the observed patterns. The parasite shows significant geographical differentiation over distances of only 1–6 km (Fricke *et al*. 2010). This suggests that gene flow is slight and should have negligible effect on allele frequencies over the years. Mutation rates for microsatellite markers are generally thought to be orders of magnitude greater than for nucleotide substitutions (Selkoe and Toonen, 2006). Although individual microsatellite alleles appear and vanish at rare frequencies in the data (Suppl. Table 1, online version only), the overall stability of allele proportions over periods of several years to 2 decades argues that mutation most likely does not account for other shifts in allele frequencies.

Distinguishing between selection and drift is a venerable challenge in evolutionary biology (Haldane, 1932; Provine, 1971; Wright, 1977). The hypothesis of co-evolution of parasite and host predicts that selection will force rapid genetic changes in the parasite over only a few generations (Price, 1980; May, 1991). Even if microsatellite markers are not linked to loci under co-evolutionary pressures, strong selection could favour only a few clones each generation, and thus drive a shift in frequencies even for unlinked loci (a genetic sweep). This would account for the rapid shifts in microsatellite allele frequencies seen for 2 species of human malaria parasites (Ferreira *et al*. 2007; Orjuela-Sanchez *et al*. 2009). Population genetic changes for the human malaria parasite are also expected to be driven by public health efforts and drug therapy used for parasite control. No such disturbance of the parasite-host system occurred for the *P. mexicanum* system. The 2-decade period of constant microsatellite allele frequencies for 2 loci observed early in the study for *P. mexicanum,* and lack of consistent rapid change for any locus throughout 3 decades, run

counter to the expectation of rapid genetic changes driven by selection.

One explanation for the patterns seen for *P. mexicanum* is the influence of drift after a drop in prevalence of the parasite, and thus a bottleneck in the effective population size (Ne) of the lizard malaria parasite at the site. But did the *P. mexicanum* Ne actually drop significantly when the parasite prevalence dropped? That is, was the drop in prevalence of a magnitude that would cause a long-term reduction in the breeding population size that would lead to unstable allele frequencies? The results are equivocal, with 2 loci showing stability until the demographic drop, and 2 showing a more gradual change in allele frequencies. Every method used to determine Ne has limiting assumptions (Wang, 2005), so we used 2 distinct methods here. Both indicated a drop in Ne following the drop in prevalence, and this agrees with results using yet a third method in a previous study on this system (Vardo and Schall, 2007). Nonetheless, the drop in prevalence from about 25% of lizards infected to about 12%, and drop in Ne by about a third seems too small to drive rapid drift. The methods used here also tend to underestimate Ne (Garza, 2005), so the later real Ne may be even greater than the estimated 32 000. The method of Garza and Williamson (2001), though, suggests that Ne did experience a drop similar to that seen for populations of free-living species that have suffered a significant demographic decline. Comparing known stable populations with those that experienced a severe bottleneck suggests that $M < 0{\cdot}70$ indicates a drop in effective population size (Garza and Williamson, 2001). Our data were consistent with a drop in effective population size for *P. mexicanum* when the prevalence declined, with a value of M between 0·41 and 0·69 depending on locus.

A final explanation for the observed patterns could be a systematic bias in determining allele frequencies. Hastings *et al*. (2010) suggested that when prevalence is high, mixed-clone infections are more common and common alleles would be under-counted, and when prevalence is low, the reverse bias would occur, with the common alleles being over-counted. No shift in number of mixed infections before and after the drop in prevalence was observed (Vardo and Schall, 2007), and the results of Hastings *et al*. (2010) suggest that only a major drop in prevalence would cause counting biases. Thus, counting errors would not easily explain the patterns seen for *P. mexicanum*.

Whatever led to changes in allele frequencies for some loci after the decline in prevalence, the effect continued over a minimum of 10 transmission cycles, a long period of genetic changes. The ability of drift to disrupt adaptive evolutionary change driven by natural selection has long intrigued biologists (Wright, 1977). These results therefore beg the question of whether important aspects of the biology of *P. mexicanum* such as life histories, transmission biology, and virulence may be disrupted each year by the continuing shifts in allele frequencies. This report argues for the value of long-term studies on parasites in their natural environments. The methodology of storing samples, and maintenance of uniform sampling methods may be taxing, but productive.

ACKNOWLEDGEMENTS

This project was possible only because of the excellent management and facilities of the University of California Hopland Research and Extension Center. We thank the staff of the HREC for more than 3 decades of warm welcome and support. Many graduate and undergraduate students collected samples over the years; we particularly thank C. Bromwich, R. Eisen, S. Osgood, A. Vardo-Zalik, A. Neal, and J. Fricke. In the laboratory, A. Vardo-Zalik, J. Fricke, J. Grauer, and A. Neal assisted with the genotyping. C. W. Kilpatrick, C. Goodnight, G. Ward, and J. Seger offered important comments.

FINANCIAL SUPPORT

The research was funded by a University of Vermont McNair Scholar Fellowship to K.S. (funded to J. Vigoreaux) and an NSF grant to J.J.S. (DEB 081383).

SUPPLEMENTARY INFORMATION

Supplementary information includes details of Supplementary Table 1. See Cambridge Journals On-Line, Parasitology.

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