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GEOGRAPHIC GENETIC DIFFERENTIATION OF A MALARIA PARASITE, *PLASMODIUM MEXICANUM*, AND ITS LIZARD HOST, *SCELOPORUS OCCIDENTALIS*

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ABSTRACT: Gene flow, and resulting degree of genetic differentiation among populations, will shape geographic genetic patterns and possibly local adaptation of parasites and their hosts. Some studies of *Plasmodium falciparum* in humans show substantial differentiation of the parasite in locations separated by only a few kilometers, a paradoxical finding for a parasite in a large, mobile host. We examined genetic differentiation of the malaria parasite *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*, at 8 sites in northern California, with the use of variable microsatellite markers for both species. These lizards are small and highly territorial, so we expected local genetic differentiation of both parasite and lizard. Populations of *P. mexicanum* were found to be differentiated by analysis of 5 markers (F_{st} values >0.05–0.10) over distances as short as 230–400 m, and greatly differentiated (F_{st} values >0.25) for sites separated by ~10 km. In contrast, the lizard host had no, or very low, levels of differentiation for 3 markers, even for sites >40 km distant. Thus, gene flow for the lizard was great, but despite the mobility of the vertebrate host, the parasite was locally genetically distinct. This discrepancy could result if infected lizards move little, but their noninfected relatives were more mobile. Previous studies on the virulence of *P. mexicanum* for fence lizards support this hypothesis. However, changing prevalence of the parasite, without changes in density of the lizard, could also result in this pattern.

The geographic mosaic model of coevolution posits that geographically widespread interacting species, such as parasites and their hosts, may exist as a patchwork of genetically distinct local populations, provided gene flow is restricted. This population structure could allow evolution of local adaptations, such as shifts in a parasite's life history traits based on local transmission ecology and host density (Gandon et al., 1996; Tucker and Stevens, 2003; Thompson, 2005). High levels of gene flow, however, would not only preclude local adaptation, but might yield maladapted populations of both parasite and host (Thompson, 2005). Thus, understanding the geographic genetic structure of parasites is critical for ecological, epidemiological, and genetic studies, and for control efforts of the pathogens of humans, domestic animals, and endangered wildlife species.

Investigators have long suspected that malaria parasites of humans vary geographically in important life history traits, including virulence (Hackett, 1937; Rogerson and Carter, 2008), which suggests that each *Plasmodium* species represents a genetic mosaic over space. Molecular techniques now permit detailed studies on genetic differentiation of malaria parasites at several spatial scales, and give some indication of the likely degree of gene flow among sites. Surveys reveal substantial genetic differentiation among continental regions (Creasey et al., 1990; Walliker et al., 1998; Anderson et al., 2000; Conway et al., 2001; Li et al., 2001; Conway, 2007) and among isolated Pacific islands (Lum et al., 2004; Conway, 2007). Within countries, the results are more variable, with some studies identifying little or no variation among sites 30–100 km apart (Zhong et al., 2007; Prugnolle et al., 2008; Bonizzoni et al., 2009), and others finding significant within-country differentiation (Creasey et al., 1990; Machado et al., 2004; Zhong et al., 2007). Most striking are findings of genetic differentiation of *Plasmodium falciparum* for a fine spatial scale, over only 7 km in Borneo (Anthony et al., 2005; Conway, 2007), and villages in Papua New Guinea only 2–5 km apart (Forsyth et al., 1989). These results are significant for understanding the dispersal of malaria parasites via their vertebrate and insect hosts, and must be considered for planning any public health program (Walliker et al., 1998).

We approached this issue with a study of a malaria parasite of a nonhuman vertebrate host, *Plasmodium mexicanum*, a parasite of fence lizards (*Sceloporus occidentalis*) in northern California. We surveyed a series of sites from 0.23 to 43 km apart, and determined the degree of genetic differentiation of both parasite and its lizard host with the use of microsatellite genetic markers. The vectors, 2 species of psychodid sand fly, *Lutzomyia vexator* and *Lutzomyia stewardi* (Fialho and Schall, 1995), are weak fliers (at least in laboratory cages) and emerge at night only when there is little or no air movement (Schall and Marghoob, 1995). Thus, their dispersal is predicted to be slight. Gene flow for fence lizards among sites is also likely to be low because the lizards are strongly territorial, and individuals remain within a small patch of the habitat for 1–4 yr (their lifetime) (Bromwich and Schall, 1986; Sheldahl and Martins, 2000; Eisen, 2001). Previous studies have found variation in parasite prevalence among sites even a few hundred meters apart (Schall and Marghoob, 1995; Eisen and Wright, 2001), and differences in genetic diversity for sites with chronically high versus low prevalence (Vardo and Schall, 2007). Thus, the overall natural history of this system suggests that *P. mexicanum* could be genetically distinct among sites.

MATERIALS AND METHODS

The base for field studies was the University of California Hopland Research and Extension Center (HREC), located near the town of Hopland in Mendocino County, California, approximately 160 km north of San Francisco. The HREC has been the site of long-term studies on the biology of *P. mexicanum* since 1978 (Schall, 1996; Vardo-Zalik and Schall, 2008). Four sites were surveyed on the field station; these sites were from 0.23 to 0.97 km apart (Fig. 1). Four sites were chosen off the station, with overall distance from 6.4 to 42.6 km from one another and HREC (Fig. 2). Site data, including latitude and longitude, are provided in Table I. The parasite was not present in 2 of the lizard populations off station (Boonville and Keiffer Ranch), but genetic data on the lizards were included in the study. All sites were in the valleys of the coastal ranges, and had similar habitats of rolling grassy hills dominated by oaks (*Quercus* spp.) and Pacific madrone (*Arbutus menziessi*). Although sites on the HREC property were closely positioned, the steep terrain and patchy distribution of suitable lizard habitat (fallen trees, rock piles) would make movement between some sites difficult for lizards and perhaps for the sand flies.

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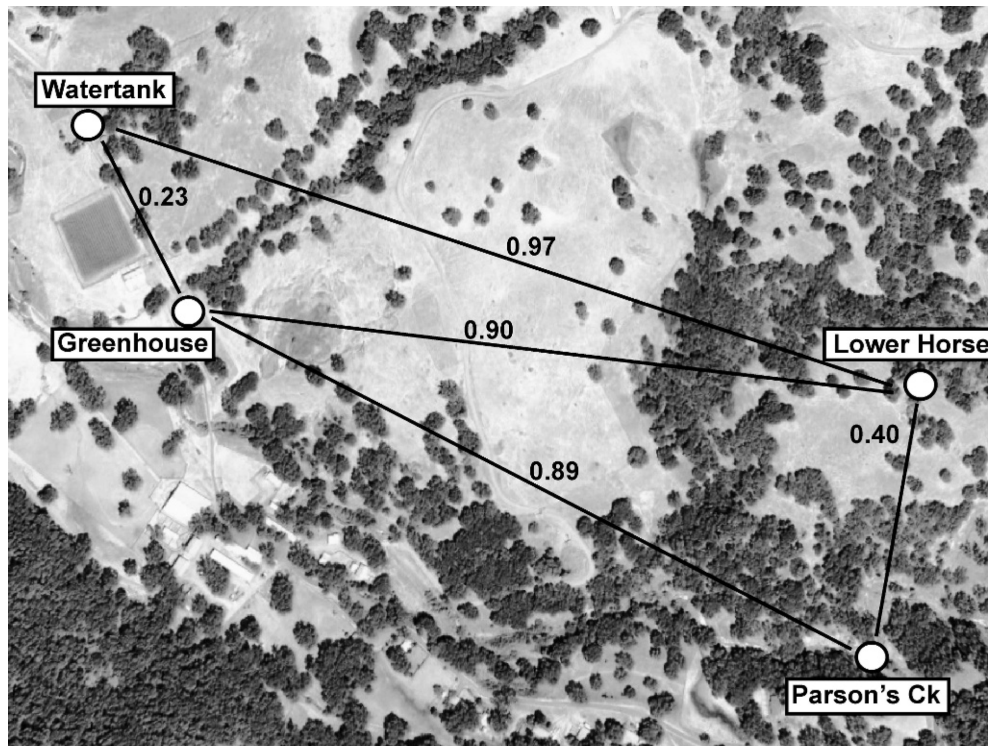

FIGURE 1. Map, with topographical features, showing 4 sites on the Hopland Research and Extension Center field station. Dark areas are individual trees, and light areas are grassland. Distances between sites are given in kilometers. Exact locations of all sites are given in Table I.

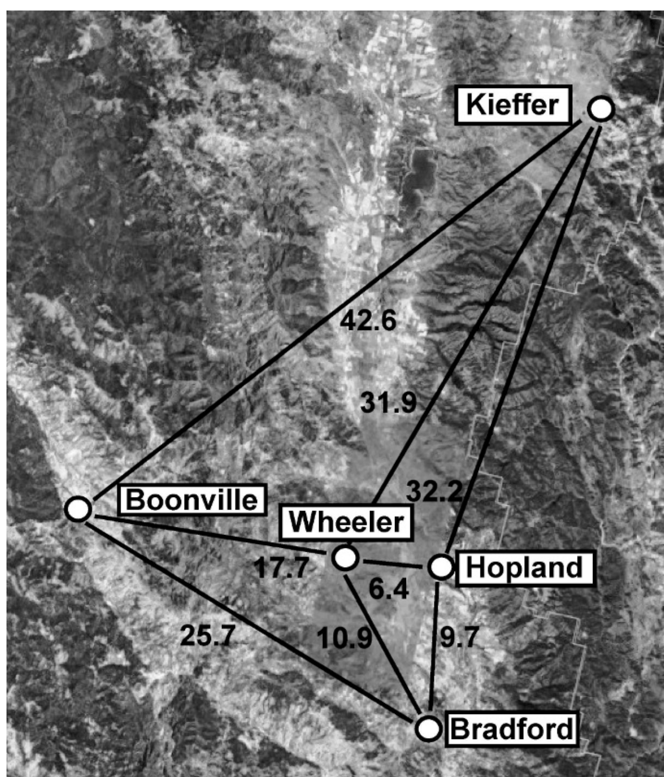

FIGURE 2. Map, with topographical features, showing 4 sites off the Hopland Research and Extension Center. Dark areas are forested mountains, and light areas are valleys. Exact locations of all sites are given in Table I. Distances between sites are given in kilometers.

Lizards were sampled by noosing with a fishing pole and a loop of nylon fishing line. The lizards were brought into the laboratory, and in the evening a toe clip allowed sampling of a few drops of blood to make a thin blood smear and dried blood dots on filter paper. The smears were stained (Giemsa stain) to identify infected lizards (scanning the smear at $\times$1,000), and the dried blood drops were stored frozen until processing. All lizards were returned to their point of capture the next morning. Sampling of lizards followed an approved animal care protocol of the University of Vermont and collecting permits from the California Department of Fish and Game.

Microsatellite markers were used to determine genetic differentiation among sites for both *P. mexicanum* and its fence lizard host. These markers are short tandem repeat regions of 1, to many, nucleotides in an organism's genome, and are very common in most species, including *Plasmodium* spp. parasites and vertebrates (Selkoe and Toonen, 2006; Schall and Vardo, 2007). The number of repeats represents a length allele. Microsatellite markers are presumed to be neutral and thus allow determination of geographic patterns in population structure (Anderson et al., 2000; Selkoe and Toonen, 2006). The 5 *P. mexicanum* microsatellite markers used for the analyses (Pmx306, Pmx710, Pmx732, Pmx747, and Pmx839) contain a tribase repeat (ATT) and were characterized by Schall and Vardo (2007). Mixed-clone infections make determination of linkage among these markers problematic, but measures of linkage disequilibrium suggest that the 5 markers are not linked (Schall and Vardo, 2007). The 3 lizard microsatellite markers used in this study were identified from a genome digest library created in the search for *P. mexicanum* microsatellites (Schall and Vardo, 2007). A linkage disequilibrium analysis performed with the use of FSTAT found no evidence of linkage among any of the lizard markers ($P > 0.05$).

DNA was extracted for both infected and noninfected lizards from the dried blood with the use of the DNeasy kit (Qiagen, Valencia, California) and the supplier's protocol. All microsatellite markers were amplified in a 25-µl PCR using Ready-to-Go beads (GE Healthcare, Piscataway, New Jersey) that contained DNA polymerase, dNTPs, and buffers; 1 µl of each 10 µM primer and 3 µl of DNA template were added to each reaction mixture.

The *P. mexicanum* microsatellite markers were amplified with the use of primers and PCR programs given in Schall and Vardo (2007), with 1

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TABLE I. GPS coordinates (in decimal degrees), elevation, and parasite prevalence (proportion of lizard host, *Sceloporus occidentalis*, infected with malaria parasite *Plasmodium mexicanum*) at sites surveyed in study. Sites in bold are those on the Hopland REC field station, and are all within 1 km pair-wise distance. Code abbreviation used in other tables is given for each site.

Site name (code abbreviation)	Elevation (m)	Coordinates	Parasite prevalence
Boonville (Boon)	175	38.9882N, 123.3653W	0/103 = 0
Bradford Ranch (BR)	223	38.9085N, 123.0848W	11/143 = 0.077
Kieffer Ranch (KR)	396	39.2876N, 123.0484W	0/55 = 0
Wheeler Ranch (WR)	272	38.9957N, 123.1601W	46/246 = 0.187
Greenhouse (GH)	256	39.0044N, 123.0863W	12/59 = 0.203
Lower Horse (LH)	383	39.0044N, 123.0760W	35/195 = 0.179
Parson's Creek (PC)	260	39.0010N, 123.0771W	31/216 = 0.144
Water Tank (WT)	262	39.0063N, 123.0869W	36/165 = 0.218

primer labeled with the 6FAM fluorescent dye. For lizard microsatellite markers, we used a labeled "tail" primer in which the same labeled oligonucleotide tail was added to 1 primer of each pair (Schuelke, 2000). Primers for 3 lizard tribase (ATT) microsatellite markers were: Soc840 (F: CAG TCG GGC GTC STC AGT GGA ACC AGA TTG GTT TCT G; R: GTA ACA AGT AGT TGC AAA AGG CC), Soc 857 (F: CAG TCG GGC GTC ATC SGG SST SGC GGT CTA TAA ATA AC; R: GTC CAA AAC ACA CTT TCT TG), and Soc700b (F: CAG TCG GGC GTC ATC AGT ATC TTG ATA GCT TCA AAA CCA CTT; R: ATA CCT TTT AAT GTT GTA CCG TTC GC). PCR amplification of the lizard markers used the program 94 C for 5 min, then 32 cycles of 94 C for 30 sec, 54 C for 45 sec, and 72 C for 45 sec, then 8 cycles of 94 C for 30 sec, 56 C for 45 sec, and 72 C for 45 sec, followed by a final 72 C extension of 10 min. All tail fragments were labeled with 6FAM fluorescent dye. The forward plus tail 10 μM primer was added in 1/10 μl per reaction and the reverse and labeled tail 10 μM primers were added in 1 μl in each reaction. During the lower of the 2 annealing temperatures, the annealing of the forward primer + tail was favored, and all of that primer was exhausted after 32 cycles. The last 8 cycles at 56 C preferentially favored the attachment of the 6FAM labeled tail fragment to the end of the forward primer.

PCR product was diluted based on its concentration, run through the ABI Prism 3100 genetic analyzer (Cornell University Life Sciences Core, Ithaca, New York), and the resulting pherograms examined using the GeneMapper program version 3.7 (2004, ABI). Each resulting peak (allele length) in a pherogram of a parasite microsatellite marker represented a haploid clone in the infection. For a marker, 1 peak represented a single clone present in the infection; 2 or more peaks resulted if the infection included multiple clones. All alleles (clones) were included if the peak had a characteristic shape (stutter pattern), with a minimum of 50 fluorescent units on the pherogram. A previous study verified that this method detects clones at low density in the lizard's blood (Vardo-Zalik et al., 2009), and studies on the effect of multiclone infection on the life history of *P. mexicanum* also demonstrated that these smaller peaks represented distinct clones (Vardo-Zalik and Schall, 2008, 2009). Mixed-clone infections are very common at the study area (50–80% of infections over sites) (Vardo and Schall, 2007), so it was not possible to define reliably multilocus genotypes of parasite. Therefore, each marker was analyzed separately. Details on the distribution of clone numbers among infections, changes in genetic diversity over time, etc., are presented in Vardo and Schall (2007).

For each microsatellite marker from the lizard genome, 1 peak represented a homozygote for that allele, and 2 peaks indicated the lizard was a heterozygote. For uniformity in method of analysis, the lizard data were also examined by marker.

Diversity of microsatellite length alleles was determined in 2 ways—by the number of alleles detected in the sample, and by expected heterozygosity (He = $[n/n-1]$ $(1 - \sigma p_i^2)$, where n is the number of infections and p_i is the frequency of the ith allele); this is a measure of genetic diversity that includes number of alleles and their relative proportions (Vardo and Schall, 2007). Sample sizes were number of lizards (for lizard data) and number of clones (total number of peaks seen for each locus) for all infections for the parasite.

Genetic differentiation of *P. mexicanum* and *S. occidentalis* was measured through pairwise F_{st} estimates (Weir and Cockerham, 1984; Cockerham and Weir, 1993), calculated with the program FSTAT (Goudet, 1995). Balloux and Lugon-Moulin (2002) compared the F_{st} with other methods to analyze microsatellite data, and found that F_{st} produced unbiased results and appears to be the best way to identify differentiation among sites. Defining a cut-off value for F_{st} that represents biologically important genetic differentiation between sites remains subjective. We use cutoffs that are commonly used (Balloux and Lugon-Moulin, 2002): F_{st} of 0–0.05 means no, or very little, differentiation, and we regard this as "no differentiation"; 0.05–0.15 means "moderate differentiation"; 0.15–0.25 is "great differentiation"; and $F_{st} > 0.25$ is "very great differentiation." Although samples were available for most sites from 1997 to 2008, a preliminary analysis revealed that microsatellite allele frequencies for *P. mexicanum* have changed over that time, so only the samples from 2006 to 2008 were used for this analysis among sites. No differences in allele frequency were found among these samples (pairwise by year, FSTAT analysis; data not shown).

RESULTS

All microsatellite markers used here for both parasite and lizard revealed substantial variation, both in number of alleles and genetic diversity measured by expected heterozygosity (Table II) and, therefore, are useful in measuring degree of genetic differentiation among sites. *Plasmodium mexicanum* was found infecting lizards at all Hopland REC sites and at 2 of the more distant sites; no infected lizards were identified from Boonville or Keiffer Ranch (Table I).

For *P. mexicanum*, of 75 comparisons of F_{st} values for all sites and all markers (total cells for *P. mexicanum* results in Table III), 34 (45%) were genetically differentiated, with $F_{st} > 0.05$. For sites >6 km apart, 27/45 (60%) were genetically differentiated, with F_{st}

TABLE II. Sample sizes among sites (range), and number of alleles detected and expected heterozygosity (He), a measure of genetic diversity for each microsatellite marker used in the study (median and ranges). The sample sizes for the parasite are based on the total number of peaks seen on the pherograms for each marker at each site; for the lizard, sample sizes are for the number of individual lizards sampled.

	N	N alleles	He
Parasite			
Pmx306	14–54	10 (5–18)	0.867 (0.696–0.912)
Pmx710	7–49	10 (4–14)	0.708 (0.696–0.912)
Pmx732	9–52	9.5 (3–16)	0.598 (0.189–0.767)
Pmx747	11–47	7 (4–10)	0.576 (0.396–0.792)
Pmx839	11–49	10 (6–16)	0.723 (0.667–0.828)
Lizard			
Soc700	28–34	8.5 (7–10)	0.826 (0.797–0.849)
Soc840	23–34	10 (7–11)	0.824 (0.783–0.878)
Soc857	28–35	9 (8–12)	0.825 (0.769–0.871)

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TABLE III. Genetic differentiation of *Plasmodium mexicanum* over 6 sites in northern California. Site locations and code are given in Table I. Pairwise F_{st} values are given for 5 microsatellite markers (Materials and Methods).*

	LH	GH	WT	WR	BR
Pmx306					
PC	0.0579	0.0137	0.0290	0.0107	0.0586
LH		0.002	0.0265	0.0260	0.1810
GH			<0.001	<0.001	0.0913
WT				0.0086	0.0604
WR					0.0815
Pmx710					
PC	0.0408	0.010	0.0140	0.0110	0.0456
LH		<0.001	0.0306	0.0131	0.1484
GH			<0.001	<0.001	0.0562
WT				0.0084	0.0287
WR					0.0811
Pmx732					
PC	0.0335	0.0972	0.0551	0.0244	0.2370
LH		0.0173	<0.001	0.1011	0.3803
GH			0.0011	0.1457	0.4316
WT				0.1303	0.4079
WR					0.1268
Pmx747					
PC	0.0896	0.0019	0.0772	0.0319	0.0165
LH		0.0514	0.0336	0.0923	0.2239
GH			0.0880	0.0689	0.1199
WT				0.1023	0.1932
WR					0.0301
Pmx839					
PC	<0.00	< 0.001	<0.001	0.0227	0.1019
LH		< 0.001	0.0183	0.0275	0.1142
GH			0.0070	<0.001	0.0965
WT				0.0158	0.0561
WR					0.0717

* Sites are LH, Lower Horse; GH, Greenhouse; WT, Water Tank; WR, Wheeler Ranch; BR, Bradford Ranch; PC, Parson's Creek.

TABLE IV. Genetic differentiation of fence lizard *Sceloporus occidentalis* over 7 sites in northern California. Site locations and code are given in Table I. Pairwise F_{st} values are given for microsatellite markers (Materials and Methods).

	LH	GH	WT	WR	BR	Boon	KR
Soc700							
PC	0.015	<0.001	<0.001	<0.001	0.0170	<0.001	<0.001
LH		<0.001	<0.001	<0.001	0.0190	0.0050	<0.001
GH			<0.001	<0.001	0.0240	0.0195	0.0037
WT				0.0050	0.0170	0.0185	<0.001
WR					<0.001	<0.001	<0.001
BR						0.0101	0.0056
Boon							<0.001
Soc840							
PC	<0.001	<0.001	<0.001	0.0136	0.0150	0.0138	<0.001
LH		<0.001	<0.001	0.0278	0.0207	0.0182	<0.001
GH			0.0101	0.0303	0.0250	0.0031	<0.001
WT				0.0322	0.0205	0.0130	<0.001
WR					0.0477	0.0469	0.0262
BR						0.0092	0.0261
Boon							0.0024
Soc857							
PC	0.0082	0.0011	0.0232	0.0385	0.0089	0.0201	0.0441
LH		<0.001	0.0004	0.0415	0.0245	<0.001	0.0716
GH			0.0324	0.0379	<0.001	<0.001	0.0434
WT				0.0736	0.0485	0.0173	0.1041
WR					0.0382	0.0453	<0.001
BR						0.0143	0.0335
Boon							0.0670

* Sites are LH, Lower Horse; GH, Greenhouse; WT, Water Tank; WR, Wheeler Ranch; BR, Bradford Ranch; Boon, Boonville; KR, Kieffer Ranch; PC, Parson's Creek.

values ranging from 0.0562 to 0.4316. For Bradford Ranch, of 25 comparisons with other sites, 13 showed moderate differentiation from other sites, 5 with great differentiation, and 3 with very great differentiation. Even on the Hopland station itself, there was an indication of differentiation between sites <1 km apart (Table III; Fig. 1). The topography between these sites is complex and steep, and suitable areas for lizards are separated by steep, grassy slopes.

Results for the lizard host revealed a different pattern, with little evidence for differentiation among sites, except for those furthest apart (Table IV). Of 84 comparisons for all sites and markers, only 4 were scored as differentiated (5%), and all of these were for a single marker (Soc857). These measures of moderate differentiation were for sites most distant, >6 km apart (4 of 63 such comparisons, or 6%).

DISCUSSION

Genetic differentiation among sites was determined with the use of variable microsatellite markers for a malaria parasite *P. mexicanum* and its lizard host *S. occidentalis*. With the use of pairwise F_{st} measures, the genetic diversity of both *P. mexicanum* and the lizard for sites from very nearby (200 m) to 40+ km was examined. The goal of this project was to test the hypothesis that low movement of the small territorial vertebrate host and its sand-fly vector should result in genetic differentiation of both lizard and parasite among sites (even those fairly nearby). Low levels of gene flow would thus allow the potential for local adaptation by both host and parasite to coincide with already observed differences in prevalence patterns (and thus likely transmission intensity) among sites (Schall and Marghoob, 1995; Eisen and Wright, 2001). The results, though, were unexpected, with the parasite found differentiated even for sites <1 km apart, but little, or no, differentiation for its vertebrate host. Thus, gene flow for the lizard is substantial, even over distances as great as 40+ km, but, despite this mobile host, the parasite remains locally distinct. The results suggest there is little opportunity for local adaptation to the parasite for fence lizards, but the parasite could well be selected for locally adaptive characters such as life history traits to match prevailing transmission intensity and lizard densities.

Our results resemble those for several studies of human malaria parasites. Anthony et al. (2005) examined the genetic structure (using microsatellite markers) of *P. falciparum* in villages in Borneo separated from <4 to 7 km. F_{st} values were associated with distance, rising from moderate differentiation for villages <4 km to very great differentiation at 7 km (for the standards used here). Forsyth et al. (1989) examined variation in the s-antigen serotypes of *P. falciparum* over even smaller distances,

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villages only 2–5 km apart in Papua New Guinea. They found substantial differentiation among villages. These results contrast sharply with other studies that find little variation for sites even 100 km apart (Zhong et al., 2007; Prugnolle et al., 2008; Bonizzoni et al., 2009). The latter case is expected because even if the vectors move little, people are mobile and would transport the parasite long distances. What would account for the very local differentiation of *P. mexicanum* when its vertebrate host experiences substantial gene flow, and could the same process that drives local differentiation in *P. mexicanum* account for the finding of local differentiation of *P. falciparum* in some situations?

When infected with *P. mexicanum*, fence lizards suffer a broad range of hematological, physiological, reproductive, and behavior deficits (Schall et al., 1982; Schall, 1990; Dunlap and Schall, 1995; Schall, 1996). Of particular relevance here are the behavioral changes in infected lizards. Infected lizards are less socially active (Schall and Sarni, 1987), and less active in their territory (Schall and Houle, 1992), and males are poor competitors for access to females (Schall and Dearing, 1987). Thus, infected animals are less likely to move any substantial distance, but their close relatives, carrying the same microsatellite alleles, may move to new sites. In mark–recapture studies (Bromwich and Schall, 1986), we occasionally found a marked animal as far as 1 km from its original home range only 24 hr after sighting the animal. Thus, gene flow can be substantial for the lizard, whereas the parasite would remain behind in its much less mobile host. The same could be true for *P. falciparum* infecting humans in Borneo and Papua New Guinea. Travel in these locations may be difficult and health care facilities not generally available, so infected people may remain at their home villages, especially when parasites are present in the blood and able to be transmitted.

The local differentiation of parasite, but not host, could derive from changing prevalence of the parasite over time rather than lack of movement of infected lizards. The prevalence of *P. mexicanum* at the HREC sites has changed substantially over the past 3 decades, with consequences for its overall genetic diversity (Vardo and Schall, 2007); allele frequencies may also have changed (see Materials and Methods section). Although not measured, the population density of the lizard appears to have remained high at all sites and constant over the past decades (our subjective judgment). Thus, shifts in the prevalence of the parasite could have led to stochastic genetic changes and the resulting differences among sites.

Studies on the genetic diversity and geographic differentiation of malaria parasites infecting wildlife hosts have lagged behind similar studies on the parasites of humans. Such studies are possible only after variable genetic markers are characterized for the parasite. Full-genome sequencing of the human malaria parasites has revealed many useful genetic markers (Anderson et al., 2000). Methods are now available for discovery of microsatellite markers of the broader diversity of malaria parasites that infect primarily birds and lizards (Schall and Vardo, 2007). The study of genetic structure of a broader range of malaria parasites will open windows into their general biology and evolution.

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