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Lack of Sequence Variation of the Mitochondrial Cytochrome *b* Gene From a Malaria Parasite, *Plasmodium mexicanum*

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ABSTRACT: Very slight sequence differences in the mitochondrial cytochrome *b* gene, even single nucleotide substitutions, have been proposed as indicative of different species of avian malaria parasites. However, few studies have examined within-species variation in that gene for *Plasmodium* or related genera. We examined sequences for the entire cytochrome *b* gene from *Plasmodium mexicanum*, a parasite of lizards, for sites where microsatellite markers revealed substantial genetic diversity. For sites where the parasite is geographically genetically differentiated, and may have been isolated for thousands of years, there was no sequence variation (1,153 nucleotides) for >160 infections studied. The low degree of variation found in the cytochrome *b* gene for two human malaria parasites world-wide, as well as the lack of variation for *P. mexicanum*, contrast with the substantial variation found in surveys of bird malaria parasites, even in restricted geographic regions.

Sequence data from the haemosporidian mitochondrial genome, and especially from the cytochrome *b* gene, have proven useful in phylogenetic studies, both within genera and in broader studies covering deeper evolutionary relationships (Escalante et al., 1998; Perkins and Schall, 2000; Perkins et al., 2007; Martinsen et al., 2008; Perkins, 2008). Mitochondrial genes vary in their rate of evolutionary change among taxa (Li, 1997), but an apparently low rate of change in the haemosporidia (Korsinczky et al., 2000) allows use of these genes for resolution of even some basal phylogenetic relationships (Perkins, 2008). Apparently conflicting with this finding, broad surveys of the malaria parasites of birds (*sensu* Martinsen et al., 2008: genera *Plasmodium* and *Parahaemoproteus*) reveal substantial variation in the cytochrome *b* gene, even for parasites falling within single morphological species (Bensch et al., 2004; Beadell et al., 2006; Martinsen et al., 2006) and for surveys over limited geographic regions (Fallon et al. 2003; Bensch et al. 2007). These findings led to the suggestion that the variation in the slowly evolving gene represents a cryptic diversity of species, rather than simply variation within species, and that parasite isolates with small genetic divergence, perhaps even a single nucleotide substitution (SNP) within the cytochrome *b* gene, may represent distinct biological species. If so, there could be thousands of cryptic species of avian malaria parasite within <200 species defined by morphology (Bensch et al., 2004).

Molecular data reveal likely cases of cryptic *Plasmodium* species of lizard (Perkins, 2000), bird (Beadell et al., 2006), and human (Win et al., 2004) hosts, but the proposal of a vast, cryptic diversity of avian malaria parasites presents a challenge for systematic parasitologists. To cast light on this issue, data are needed on intraspecific variation in the cytochrome *b* gene for well-characterized parasite species that are unlikely to include cryptic taxa. Such data are available for 2 *Plasmodium* species. A large, cross-continent survey of *Plasmodium falciparum* isolates found only 11 SNPs along a 1,131-nucleotide segment of the gene, and most of these SNPs were rare or geographically local in distribution (Ekala et al., 2007). Some of this variation, apparently, has a selective basis because specific nucleotide substitutions are associated with drug resistance by the parasite (Korsinczky et al., 2000). Two cross-continent surveys of *Plasmodium vivax* also found rather-limited variation (Jongwutiwes et al. 2005; Mu et al. 2005). For example, 14 SNPs were found in samples taken in Africa, Southeast Asia, and India (Mu et al. 2005).

We have surveyed the cytochrome *b* gene for *P. mexicanum*, a malaria parasite of lizards in the western United States. Cryptic species diversity within *P. mexicanum* seems unlikely. In the United States, the parasite infects only the western fence lizard (*Sceloporus occidentalis*) (it has never been found infecting any other lizard species over many years of sampling in California, where the parasite is common in some fence lizard populations; data not shown), is morphologically uniform everywhere it has been sampled, and is supported by both molecular phylogenetic and genetic data to be distinctive from other *Plasmodium* species (Perkins and Schall, 2000; Martinsen et al., 2008). It's closest relative, *Plasmodium chiricahuae*, is distinct by molecular phylogenetic analysis (Perkins and Schall, 2000) and is found in montane habitat islands in Arizona (Mahrt, 1987). The distribution of *P. mexicanum* is geographically patchy and is found in the relict Madro-Tertiary habitats of western North America. This patchy distribution results from habitat changes over the Pleistocene and, thus, the parasite can be sampled at sites that have been isolated for thousands of years (Ayala, 1970). We have surveyed numerous sites in northern California and have found the parasite in only small, geographically isolated patches of habitat. Microsatellite markers reveal that gene flow among these patches is low; sites only a few kilometers apart reveal substantial genetic differentiation for the parasite (Fricke et al., 2010).

Presented here are sequence results for 1,153 nucleotides of the mitochondrial genome, including the entire 1,130 nucleotides of the cytochrome *b* gene, for samples of infected lizards surveyed at several sites in California. Our goal was to determine the intraspecific variation in the cytochrome *b* sequence for this widespread malaria parasite among sites. The rate of molecular evolution in *Plasmodium* must depend on such factors as host life span (and thus possible duration of an infection), rate of parasite replication and number of merozoites produced, and sporogonic cycle in the vector. We suspected that *P. mexicanum* would be closer to the avian malaria parasites in these life history traits than to the two human malaria parasites surveyed to date. If the cytochrome *b* gene does, in fact, evolve so slowly that very few nucleotide substitutions represent isolated parasite species, then we expected to find very little or no variation for *P. mexicanum*, even for distant sites. However, if the substantial variation seen in isolates of bird malaria parasite simply represents within-species variation, then we expected to find such variation in the cytochrome *b* gene for *P. mexicanum*.

Site names and latitude and longitude (based on readings from a GPS instrument) are given in Table I. Three sites in northern California, near the town of Hopland (Mendocino County), included the University of California Hopland Research and Extension Center (HREC), the Bradford Ranch, and the Wheeler Ranch. The HREC has been the site of a long-term study on the parasite since 1978 (Schall, 1996). These sites are 6.4 to 10.9 km apart and were chosen because microsatellite markers reveal they are geographically distinct, with low rates of gene flow (Fricke et al., 2010). The last site, the University of California Sierra Foothills Research and Extension Center (SFREC), was more distant (156 km from HREC), near the town of Browns Valley (Yuba County). Discovery of the parasite at the SFREC is an extension of its known range (Ayala, 1970).

Lizards were collected by noosing, with a slip noose on the end of a fishing pole, and were taken to the laboratory that evening to make thin blood smears for later staining (Giemsa stain) and examination under ×1,000 to identify infected lizards. A few drops of blood were also dried on filter paper and stored dried and frozen for molecular analysis. All lizards were collected under permits from the state of California and with animal care and use permits from the University of Vermont.

DNA was extracted from the blood dots using the Qiagen DNeasy kit (Qiagen, Vanencia, California), following the provided protocol. The cytochrome *b* gene was PCR amplified in 2 fragments, with overlap that allowed the fragments to be edited to yield one 602 nt and the second 551 nt. Each reaction used Ready-to-Go PCR beads (GE Healthcare, Piscataway, New Jersey), which provided an optimized mix of DNA polymerase, dNTPs, and buffers. For a 25 µl reaction, 21.5 µl water was added to the bead, as well as 1.5 µl of the extracted DNA, and 1 µl of each primer at 10 µM. Primers for the first fragment were DW1P (TCA ACT ATG ACT TTA TTT GG) and DW3P (AGC AGT ATC ATA CCC TAA

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TABLE I. Sample locations for western fence lizards (*Sceloporus occidentalis*) infected with the malaria parasite *Plasmodium mexicanum*. Given are site names, GPS coordinates, and sample size of sequenced infections for two portions of the parasite's cytochrome *b* gene. The DW1P/DW3P sequence is a 602-nucleotide portion of the gene at its 3′ end, and the KS1/KS3 sequence is a 551-nucleotide portion of the gene at its 5′ end. The two pairs of primers amplified fragments that overlapped, with the overlapping portion edited to produce the two fragments.

Site Name	GPS	DW1P/DW3P	KS1/KS3
Hopland REC	39°00′N, 123°05′W	88	88
Wheeler Ranch	38°59′N, 123°10′W	46	26
Bradford Ranch	38°54′N, 123°05′W	27	28
Sierra Foothills REC	39°14′N, 121°10′W	5	5

AG), with PCR program 94 C for 2 min, followed by 32 cycles of 94 C for 20 sec, 46.5 C for 20 sec, 70 C for 30 sec, and a final extension of 70 C for 7 min. Primers for the second fragment were KS1 (GAT ATG TGG AGG ATA TCT TG) and KS3 (CTC AGA AAT GTC GTC TCA TC), with PCR program 94 C for 2 min, followed by 32 cycles of 94 C for 20 sec, 49 C for 20 sec, 70 C for 30 sec, and a final extension of 70 C for 7 min. PCR product was sequenced by a commercial facility (Agencourt, Danvers, Massachusetts) using the DW1P and KS1 primers using Big Dye technology and the ABI genetic analyzer instrument. Data were visualized using the FinchTV program (Geospiza, Seattle, Washington) and aligned using MacClade (Sinauer, Sunderland, Massachusetts). All sequences could be readily aligned, with no indels or other discrepancies. Any samples that revealed possible variation at any of the sites were sequenced again using the reverse primers (DW3P or KS3) at a second facility (Vermont Cancer Center, Burlington, Vermont).

Table I presents sample sizes for each segment sequenced for each site, for a total of 168 for the first fragment and 147 for the second. Three sequences presented a single site with double peaks, suggesting the presence of a polymorphism and mixed infection with 2 haplotypes. These were sequenced again at the second facility, using the reverse primers several times, and the results indicated that the double peaks seen originally were a sequencing artifact. Otherwise, all sequences were identical for the complete 1,130 nucleotides of the cytochrome *b* gene and for the 23 additional nucleotides at the 5′ and 3′ ends of the gene. The set of identical sequences recovered here were compared with the same portion of the mitochondrial genome deposited in Genbank (NC_009960) and were found to be identical to that sequence.

In summary, we present data surveying for within-species variation in the cytochrome *b* gene for a malaria parasite and a finding of no variation in the gene. The geographic locations where the parasite was sampled were sites as distant as 156 km apart, which would be insignificant for avian or human malaria parasites, but likely to represent genetically isolated sites for a small, less-mobile vertebrate host. The patchy distribution of suitable habitat for the parasite suggests that our sites had been isolated for a long period, at least thousands of years (Ayala, 1970). Variation was lacking, even in third-position sites in the gene sequence that would represent silent substitutions that should accumulate more rapidly than in coding sites.

Lack of variation in the cytochrome *b* gene could be a result of either a potent selective sweep or a very recent geographic expansion of the parasite that led to an essentially clonal genetic structure. Such events have been proposed to account for a suggested low genetic diversity for *P. falciparum* (Tibayrenc and Ayala, 2002). Three lines of evidence argue that *P. mexicanum* is not genetically single-clone, within and among sites, and is genetically diverse. First, microsatellite markers reveal substantial diversity, with 50–80% of infections multi-clonal, depending on prevailing prevalence levels among sites (Vardo and Schall, 2007). Second, microsatellite markers reveal that the parasite is geographically distinct among sites even a few kilometers apart (Fricke et al., 2010). Last, studies that establish experimental infection with differing specific microsatellite alleles and diversity of alleles (interpreted as distinct clones and thus variation in clonal diversity) show different life history characteristics, including rate of replication, final parasite density, and virulence (Vardo-Zalik and Schall, 2008, 2009). Thus, based on microsatellite markers, important genetic loci, most likely in the nuclear genome, that influence life history traits vary among clones identified. We also find it unlikely that *P. mexicanum* has experienced a selective sweep followed by recent geographic expansion. The lizard host is small, and movement of the lizard across currently unsuitable habitat would be difficult. Also, the insect hosts, 2 species of psychodid sandfly (*Lutzomyia* spp.), are weak fliers and are not active at night under the windy conditions that could transport infected sandflies long distances (Schall and Marghoob, 1995; Schall, 1996).

Variation in the cytochrome *b* gene for avian malaria parasites is substantial and led to the conclusion that the very small variation in the gene represents a distinct evolutionary lineage, perhaps even a species (Bensch et al., 2004). For example, lineages with $<1\%$ sequence difference are often restricted to a single bird species (Bensch et al., 2000; Ricklefs and Fallon, 2002; Waldenstrom et al., 2002). Our results add to the meager data on intraspecific variation in the cytochrome *b* gene for *Plasmodium* species. The low level of variation seen in *P. falciparum* and *P. vivax* worldwide, with at least some of that variation driven by drug-pressure selective forces (Korsinczky et al., 2000), and the lack of variation in *P. mexicanum*, does not conflict with the conclusion that small sequence differences seen in local regions for avian malaria parasites represent genetically isolated species. Clearly, within-species surveys for avian malaria parasites are needed, but such studies have a logical challenge, i.e., variation within a morphological species could be viewed either as an indication of cryptic species diversity or as within-species variation. Thus, a well-characterized species with a single host species, such as the *P. mexicanum* studied here, should be the model for such a study.

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