Detection of a Malaria Parasite (Plasmodium mexicanum) in Ectoparasites (Mites and Ticks), and Possible Significance for Transmission

Authors: Schall, JosJ., and Smith, Thomas C.

Source: Journal of Parasitology, 92(2) : 413-415

Published By: American Society of Parasitologists

URL: https://doi.org/10.1645/GE-688R.1

J. Parasitol., 92(2), 2006, pp. 411–413

Systematic Position and Relationships of *Paracreptotrematina limi*, based on Partial Sequences of 28S rRNA and Cytochrome *c* oxidase Subunit 1 Genes

Christopher S. Platta and Anindo Choudhury, Division of Natural Sciences, St. Norbert College, DePere, Wisconsin 54115. *e-mail: anindo.choudhury@snc.edu*

ABSTRACT: *Paracreptotrematina limi* Amin and Myer, 1982 (Trematoda), an intestinal fluke specific to the mudminnow, *Umbra limi*, is conventionally classified within the papillose Allocreadiidae. Its unusual morphology (lack of identifiable vitellaria, large fully embryonated terminal eggs), assumptions of homology of its 2 atypical muscular oral 'papillae' (lobes) with those of the Bunoderinae, and its unknown life cycle make this classification tenuous. Previous phylogenetic analyses of the papillose allocreadiids, based on morphology, placed *P. limi* as a basal papillose allocreadiid. We tested this hypothesis with a phylogenetic analysis by using partial sequences of the 28S ribosomal RNA gene and the cytochrome *c* oxidase subunit I gene from several plagiorchiiform taxa, including reportedly related allocreadiids as well as selected species of Plagiorchiidae, Haematoloechidae, and Macroderoididae. Results of phylogenetic analyses of the 28S rRNA gene fragments by using parsimony criteria support the classification of *P. limi* as an allocreadiid and place it as a sister taxon to a clade with *Allocreadium lobatum* Wallin, 1909, *Bunodera luciopercae* (Müller, 1876) and *Crepidostomum cooperi* Hopkins, 1931, with *Polylekithum ictaluri* (Pearse, 1924) basal to all of them. Analysis of the cytochrome *c* oxidase subunit I gene sequence data from fewer taxa supports the placement of *P. limi* relative to 3 (*A. lobatum*, *C. cooperi*, *and P. ictaluri*) of the 4 allocreadiid taxa. These results also suggest that the previous conception of the papillose allocreadiids as a monophyletic assemblage that includes *P. limi* may require a reappraisal.

Paracreptotrematina limi Amin and Myer, 1982 is an intestinal trematode specific to its host, the mudminnow, *Umbra limi* (Kirtland). The parasite is currently classified within Allocreadiidae (Amin and Myer, 1982; Caira, 1989). Its unusual morphology includes rather unique looking oral muscular papillae (compared with other papillose allocreadiids), the absence of clearly identifiable vitellaria, and very large fully embryonated terminal eggs that hatch immediately when eggs are released from the uterus into water (A. Choudhury, unpubl. obs.). Assumptions of the homology of its 2 muscular oral 'papillae' (lobes) with the ventral oral lobes of the Bunoderinae and its unknown life cycle, make the classification of *P. limi* as an allocreadiid tenuous. The position of the uterus as it winds in between the oblique testes without obscuring them also resembles that of the macroderoidids. Furthermore, it has been placed in its genus with another species, *Paracreptotrematina aguirrepeqenoi* (Guzman, 1973) in Mexico, with which it shares a superficial resemblance. To date, nothing is known about its life cycle and there is no other freshwater trematode that is readily recognizable as being similar to it. A previous phylogenetic analysis of 'papillose allocreadiids' suggested a basal position for *P. limi* (Caira, 1989), within this group, but this placement and that of other 'papillose allocreadiids' was based on the number of muscular oral papillae present.

The unique combination of morphological characters in *P. limi*, the lack of knowledge of its life cycle, and the limited data available for a morphology-based phylogenetic analysis, prompted a molecular study on the phylogenetic relationships of *P. limi*, especially in relation to other allocreadiids and macroderoidids. Partial sequences from the 28S region of the ribosomal genome and the cytochrome *c* oxidase subunit I (COI) gene were used for a phylogenetic analysis to develop a more robust hypothesis of the relationships of this unusual trematode.

Specimens of *P. limi* were collected from mudminnows from the Brokenhead River in Manitoba, Canada, during summer 2003. Specimens of 4 other allocreadiids used in this study were collected by one of us (A.C.) as follows: *Allocreadium lobatum* Wallin, 1909, ex. creek chub, *Semotilus atromaculatus* (Mitchill), Rose Isle Creek, Manitoba, Canada; *Crepidostomum cooperi* Hopkins, 1931, ex. trout perch, *Percopsis omiscomaycus* (Walbaum), Hillside, Lake Winnipeg, Manitoba, Canada; *Bunodera luciopercae* (Müller, 1876) ex. yellow perch, *Perca flavescens* (Mitchill), Lake Sasajewun, Algonquin Park, Ontario, Canada; and *Polylekithum ictaluri* (Pearse, 1924) ex. channel catfish, *Ictalurus punctatus* (Rafinesque), Assiniboine River at Perimeter Highway Bridge, Winnipeg, Manitoba, Canada. Voucher specimens (whole mounts) of the allocreadiids used in this study are in the U.S. National Parasite Collection (USNPC), with corresponding accession numbers as follows: *P. limi* (USNPC 97076), *A. lobatum* (USNPC 97068), *C. cooperi* (USNPC 97066), *B. luciopercae* (USNPC 97071 and 97072), and *P. ictaluri* (USNPC 97067). Live trematodes were washed with saline and stored refrigerated in 95 or 100% ethanol. The 28S rRNA gene from *P. limi* and the COI gene from *P. limi*, *P. ictaluri*, and *C. cooperi* were amplified as follows: Worms were digested overnight at 37 C, by using proteinase K (200 μg/ml) in TES buffer (10 mM Tris-Cl, 1 mM EDTA, and 25 mM NaCl, pH 8.2). The digest was heated to 95 C for 7 min to inactivate the proteinase K and subsequently centrifuged before using the supernatant for amplification (modified from Gloor et al., 1993). Genes of interest were amplified using polymerase chain reaction (PCR) techniques. The 28S rRNA gene was amplified using the 28sy forward primer (5′CTA ACC AGG ATT CCC TCA GTA ACG GCG AGT3′) and the 28sz reverse primer (5′AGA CTC CTT GGT CCG TGT TTC AAG AC3′) (León-Règagnon et al., 1999). Both sets of primers have been used with success (León-Règagnon et al., 1999; León-Règagnon and Brooks, 2003). The COI gene was amplified using the COI-F forward primer (5′TTT TTT GGG GAT CCT GAG GTT TAT3′) and the COI-R reverse primer (5′TAA AGA AAG AAC ATA ATG AAA ATG3′) (V. León Règagnon, pers. comm.). PCR reactions were performed on a PerkinElmer GeneAmp 9700 thermocycler (PerkinElmer Life and Analytical Sciences, Boston, MA) by using Extaq DNA polymerase (TaKaRa Mirus Corporation, Madison, Wisconsin) in a total reaction volume of 50 μl. The amplification protocol consisted of an initial denaturing cycle of 5 min at 94 C, 40 cycles of the following: 94 C for 30 sec, 50 C for 30 sec for primer annealing, 72 C for 2 min for replication, and a final hold for elongation at 72 C for 5 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, California). Purified products were sent to Northwoods DNA, Inc. (Becida, Minnesota) for automated sequencing.

Sequences were received as both nucleotide sequences and chromatograms. Each sequence was manually edited for accuracy using ABI EditView (PerkinElmer Life and Analytical Sciences). Undetermined nucleotides in the sequences were left as ''N'' (undetermined) and treated as missing data. Partial sequences of the 28S rRNA gene and those of the COI gene, which were generated as part of this study, have been deposited to GenBank with their accession numbers as follows: *P. limi* (28SrRNA gene: DQ 189998; COI gene: DQ 190543), *C. cooperi* (COI gene: DQ 191365), and *P. ictaluri* (COI gene: DQ 191364). Partial sequences of the 28S rRNA gene from the other allocreadiid taxa used in this study are (GenBank accession numbers in parentheses): *A. lobatum* (DQ029327), *C. cooperi* (DQ029328), and *B. luciopercae* (DQ 029331) (León-Règagnon and Choudhury, following protocols in León-Règagnon et al., 1999; León-Règagnon and Brooks, 2003); *P. ictaluri* (DQ 189999, Rogelio Rosas Valdez, pers. comm.). The partial sequence of the COI gene of *A. lobatum* (DQ 195263) was obtained from V. León Règagnon (pers. comm.). A sequence of the COI gene from 1 other species, *Glypthelmins californiensis* (AY278058), was downloaded from GenBank for use in the analysis. Sequences of the 28S rRNA gene from 7 other species were downloaded from GenBank (accession numbers in parentheses) for use in the analysis: *Plagiorchis muelleri* Tkach and Sharpilo, 1990 (AF184250) (Palgiorchiidae), *Haematoloechus meridionalis* León Règagnon, Brooks and Zelmer, 2001 (AF531864) (Hae-

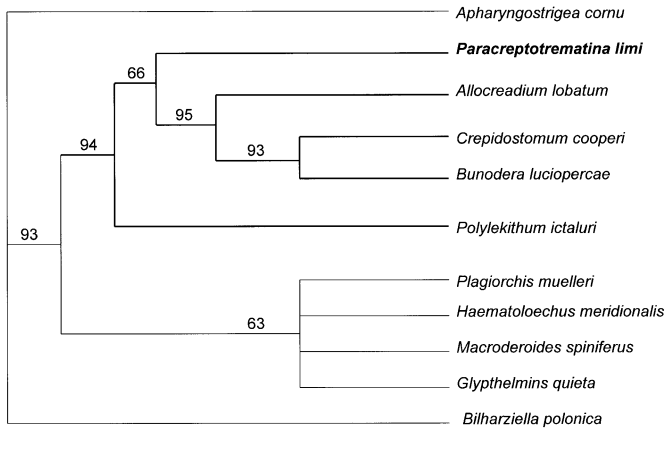

FIGURE 1. Strict consensus of 2 equally parsimonious tress from maximum parsimony analysis of partial sequences of the 28S ribosomal RNA gene, showing hypothesis of the phylogenetic relationship of *P. limi*. Numbers are bootstrap values.

matoloechidae), *Macroderoides spiniferus* Pearse, 1924 (AF433674) and *Glypthelmins quieta* (Stafford, 1900) (AY278049) (both Macroderoididae), *Apharyngostrigea cornu* (Zeder, 1800) (AF 184264) (Strigeidae), and *Bilharziella polonica* (Kowalewski, 1895) (AF 184265) (Schistosomatidae). Sequences were aligned using Clustal alignment software in MEGA 3 (Kumar et al., 2004) and checked by eye. A phylogenetic analysis was performed using PAUP* 4.0 (phylogenetic analysis using parsimony, Swofford, 2002). Branch and bound searches were performed for each individual data set (28S and COI) by using maximum parsimony analysis. The strigeid *Apharyngostrigea cornu* (AF 184264) and the schistosomatid *B. polonica* (AF 184265) were used as outgroup taxa for the 28S rRNA gene analysis, given their more basal position relative to the 'Plagiorchiida' (Olson et al., 2003). Of 382 total characters used in the 28S rRNA gene analysis, 83 were parsimony-informative. For the analysis based on partial sequences of the COI gene, fewer relevant taxa were available. This analysis was therefore only used to test the relationship of *P. limi* to the 3 allocreadiids (*A. lobatum*, *P. ictaluri*, and *C. cooperi*) for which COI sequence data were available, and the macroderoidid *G. californiensis* was used as the outgroup. Of the 361 total characters used in the COI analysis, 51 were parsimony-informative. Bootstrap analysis was conducted on both 28S and COI data sets by using 1,000 replicates.

A maximum parsimony analysis of the partial sequences of the 28S rRNA gene yielded 2 equally parsimonious trees, 257 steps long, with a CI of 0.716 and an RI of 0.628. A strict consensus of the 2 trees (Fig. 1) shows *P. limi* grouping with the 4 other allocreadiids (*A. lobatum*, *C. cooperi*, *B. luciopercase* and *P. ictaluri*) used in the study. Nodal support indicates a bootstrap value of 94 for this relationship. *Paracreptotrematina limi* was more closely allied with the allocreadiids than any of the macroderoidids used in the study. There was no difference between the two equally parsimonious trees in the relationship of *P. limi* to other allocreadiids used in the analysis. A maximum parsimony analysis of the mitochondrial data set yielded 1 tree, 308 steps long, with a consistency index (CI) of 0.896, and a retention index (RI) of 0.373, in which *P. limi* grouped with *C. cooperi* and *A. lobatum*, with *P. ictaluri* basal to them (Fig. 2). The relationship of *P. limi* to *C. cooperi*, and *A. lobatum* was supported by a bootstrap value of 66.

This is the first time that *P. limi* has been studied genetically, and the first time that sequences from portions of its genome have been generated. In addition, this is also one of the few reports of a phylogenetic analysis of allocreadiid genera by using molecular data (see GenBank, www.ncbi.nlm.nih.gov). Molecular analysis of the 28S rRNA data set supports the placement of *P. limi* in the family Allocreadiidae and not Macroderoididae. The molecular data further support the hypothesis from morphology-based phylogenetic analyses (Caira, 1989) that *P. limi* occupies a relatively basal position among the papillose allocreadiids.

Our study also raises some potential problems with the assumed

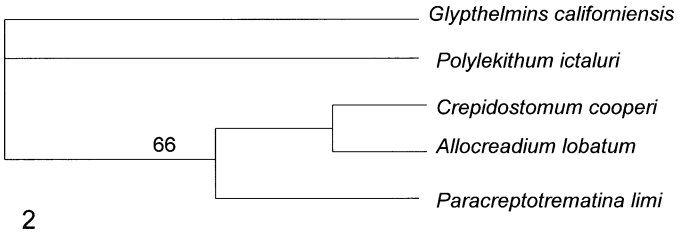

FIGURE 2. Hypothesis of phylogenetic relationships of *P. limi* to 3 allocreadiids, based on partial sequences of the COI gene. Number indicates bootstrap value.

monophyly of the 'papillose allocreadiids'. Based on morphology, *P. limi*, *C. cooperi*, and *B. luciopercae* are conventionally considered papillose allocreadiids (Hopkins, 1934; Caira, 1989), whereas *A. lobatum* and *P. ictaluri* are not. Yet, the analyses (Fig. 1) suggest that the clade *Crepidostomum* + *Bunodera* is more closely related to *A. lobatum* than it is to *P. limi*, and the COI data provide some support for this hypothesis (Fig. 2). This means that either *A. lobatum* has lost the muscular papillae or that the ventral papillae of *P. limi* are not homologous to the papillae of *C. cooperi*, in which case *P. limi* is not a papillose allocreadiid in the same natural way as the bunoderines. If the former is true, it also suggests that the papillose allocreadiids may not comprise a natural monophyletic assemblage without *Allocreadium*, which was not included in the original analysis of Caira (1989). This study also suggests that an analysis of the papillose allocreadiids without including nonpapillose forms such as *Allocreadium* may be incomplete. Life cycle studies on *Allocreadium isoporum* Looss 1900, a parasite of leuciscine cyprinids in Europe, and a species that is morphologically very similar to *A. lobatum* (also a parasite of cyprinids but in North America) indicate close relationships with the bunoderines (Hopkins, 1934; Yamaguti, 1975; Moravec, 1992). As noted above, the absence of any information on the life cycle of *P. limi* precludes any further statement about its similarity with the other allocreadiids. Finally, the basal placement (Fig. 1) of *P. ictaluri*, a non-'papillose' allocreadiid, further supports the notion that the simple presence or absence of the muscular oral 'papillae' may not be a robust indicator of relationships. Additional data from a wider range of allocreadiid species are necessary to provide more robust hypotheses of relationships among the 'papillose' allocreadiids.

We thank Patrick Nelson, Department of Zoology, University of Manitoba, Winnipeg, Manitoba, Canada, and Craig Walker, ProAm Bait and Tackle, Winnipeg, Manitoba, for help with obtaining mudminnows. Special thanks go to Nick Schill, Cellular and Molecular Biology Program, University of Wisconsin-Madison, for initial training of C.P., and to Russ Feirer, St. Norbert College, for advice and use of laboratory facilities. We also thank Jason Duncan, Howard Hughes Institute, San Diego, California, for advice regarding techniques and for introducing us to the single fly DNA preparation (Gloor et al., 1993). Finally, we are grateful to Rogelio Rosas Valdez and Virginia León-Règagnon, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico, for providing some of the sequences for this study. We are especially indebted to Virginia León-Règagnon for advice on various aspects of molecular systematics. This research was funded by an SNC Student-Faculty collaborative grant to A.C. and C.P. as well as the 2004 Anselm M. Keefe Fellowship to C.P.

LITERATURE CITED

AMIN, O. M., AND D. G. MYER. 1982. *Paracreptotrematina limi* gen. et sp. nov. (Digenea: Allocreadiidae) from the mudminnow, *Umbra limi*. Proceedings of the Helminthological Society of Washington **49:** 185–188.

CAIRA, J. N. 1989. A revision of the North American papillose Allocreadiidae (Digenea) with independent cladistic analyses of larval and adult forms. Bulletin of the University of Nebraska State Museum **11:** 1–58.

GLOOR, G. B., C. R. PRESTON, D. M. JOHNSON-SCHLITZ, N. A. NASSIF, R. W. PHILLIS, W. K. BENZ, H. M. ROBERTSON, AND W. R. ENGELS.

1993. Type I repressors of P Element Mobility. Genetics **135:** 81–95.

HOPKINS, S. H. 1934. The papillose Allocreadiidae. Illinois Biological Monographs **13:** 6–80.

KUMAR, S., K. TAMURA, AND M. NEI. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5:** 150–163.

LEÓN-RÈGAGNON, V., AND D. R. BROOKS. 2003. Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plachiorchiidae), with emphasis on North American species. Journal of Parasitology **89:** 1206–1211.

———, D. R. BROOKS, AND G. PEREZ-PONCE DE LEÓN. 1999. Differentiation of Mexican species of *Haematoloechus* Loss, 1989 (Digenea: Plagiorchiformes): Molecular and morphological evidence. Journal of Parasitology **85:** 935–946.

MORAVEC, F. 1992. Observations on the bionomy of *Allocreadium isoporum* (Looss, 1894) (Trematoda: Allocreadiidae). Folia Parasitologica **29:** 133–144.

OLSON, P. D., T. H. CRIBB, V. V. TKACH, R. A. BRAY, AND D. T. J. LITTLEWOOD. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal of Parasitology **33:** 733–755.

SWOFFORD, D. L. 2002. PAUP*: Version 4.0 beta 10. Sinauer Associates Inc., Sunderland, Massachusetts.

YAMAGUTI, S. 1975. A synoptical review of life histories of digenetic trematodes of vertebrates. Keigaku Publishing Co. Tokyo, 219 p.

J. Parasitol., 92(2), 2006, pp. 413–415

Detection of a Malaria Parasite (*Plasmodium mexicanum*) in Ectoparasites (Mites and Ticks), and Possible Significance for Transmission

Jos. J. Schall and Thomas C. Smith, Department of Biology, University of Vermont, Burlington, Vermont 05405. *e-mail: jschall@zoo.uvm.edu*

ABSTRACT: Two species of sandflies (*Lutzomyia*) are competent vectors of *Plasmodium mexicanum*, a malaria parasite of lizards. The very patchy distribution of sites with high *P. mexicanum* prevalence in the lizards, and often low or even nil sandfly density at such sites, provoked an evaluation of 2 common lizard ectoparasites, the tick *Ixodes pacificus* and the mite *Geckobiella occidentalis*, as potential passive vectors. *Plasmodium* sp.-specific polymerase chain primers were used to amplify a long segment of the mitochondrial cytochrome *b* gene that is unlikely to survive intact if the parasite cells are killed within a blood-feeding arthropod. The segment was strongly amplified from sandflies (the positive control for the method) from 1 to 96 hr postfeeding on an infected lizard. For ticks, the gene fragment was poorly amplified at 0 hr postfeed, and not amplified after 2 hr. In contrast, strong amplification of the parasite DNA was observed from mites from 0 to 20 hr postfeed, and weak amplification even at 96 hr.

Plasmodium mexicanum, a malaria parasite of western fence lizards (*Sceloporus occidentalis*) in California, is the only known species of *Plasmodium* that is transmitted by a vector other than a mosquito. Compelling evidence indicates that 2 species of sandflies (*Lutzomyia vexator* and *Lutzomyia stewarti*) are competent vectors for the parasite. First, the parasite completes normal development in the sandflies, producing mature oocysts on the midgut and sporozoites that travel to the salivary glands (Ayala and Lee, 1970; Fialho and Schall, 1995). Second, laboratory experiments successfully transmit the parasite by injection of sporozoites into uninfected lizards (Ayala, 1971; Fialho and Schall, 1995) or allow infected sandflies to take a blood meal on an uninfected lizard (Klein et al., 1987). Last, the parasite has evolved a mechanism to manipulate the sandfly's thermoregulatory behavior to raise the vector's temperature to one that is optimal for the development of *P. mexicanum* (Fialho and Schall, 1995), indicating a close association of the sandflies and parasite.

Despite this evidence, the transmission ecology of the parasite remains an open and vexing question. At a long-term study site in northern California (Schall, 2002a), prevalence of the parasite can be high in its lizard host, with up to 50% of lizards infected at some local areas. The vectors, though, are not abundant, and in some areas where the parasite prevalence is high, vectors are not detectable using standard survey methods (Schall and Marghoob, 1995; J. Schall and T. Smith, pers. obs.). Most problematic is the very patchy distribution of high versus low prevalence areas. Eisen and Wright (2001) found over a 3-yr period that high prevalence patches of only a few hundred square meters may be surrounded by areas with substantially lower prevalence, with groundcover being a strong predictor of prevalence. These results hint that there may be other vectors of *P. mexicanum*. For example, the very local distribution of the parasite matches trends in density of ticks and mites at the Hopland site (Talleklint-Eisen and Lane, 1999; Schall et al., 2000).

Because the sandflies are the only known biting insects to feed on fence lizards at the site, we chose to focus on 2 common ectoparasites of the lizards, the mite *Geckobiella occidentalis* and the tick *Ixodes pacificus* (Schall et al., 2000). Another apicomplexan parasite of the lizards, *Schellackia occidentalis*, is transmitted by the mite and prevalence of this parasite is higher in the fence lizards than *P. mexicanum* (data not shown). *Schellackia occidentalis* does not replicate or undergo development within the mite, but is passively transmitted when a lizard eats a mite that has very recently fed on an infected lizard (Bonorris and Ball, 1955; Klein et al., 1988). We regarded the ectoparasites as unlikely passive vectors of *P. mexicanum*, and thus sought a rapid method to eliminate them from further consideration.

We used a technique to determine whether *P. mexicanum* can be detected in the ectoparasites for periods from 0 to 96 hr after they ceased feeding on the blood of infected lizards. Polymerase chain reaction (PCR) primers that are specific to species of *Plasmodium* and related parasites, but will not anneal to any region of the arthropod genome (or that of the lizard or of *Schellackia occidentalis*), were used to amplify a moderately long DNA segment of the cytochrome *b* gene (1,174 base pairs [bp]). The parasite must survive in a competent passive vector, and thus the long DNA segment would remain intact, but in an ectoparasite that is not a suitable passive vector, the parasite would be killed when the blood meal is digested and the long segment would most likely not survive. Studies on the survival of long DNA segments from a parasite in known unsuitable versus competent vectors are not available for comparison. However, in support of the method used here, many studies have detected DNA from the blood of the vertebrate in blood-feeding arthropods. Only short DNA segments from a target gene (for example, 300 bp for the cytochrome *b* gene) can be detected (Lee et al., 2002; Malmqvist et al., 2004), and quality of the DNA drops sharply within a few hours during digestion by the arthropod (Chow-Shaffer et al., 2000; Lee et al., 2002; Mukabana et al., 2002).

The study was conducted at the University of California Hopland Research and Extension Center, a 2,169 ha property near the town of Hopland in southern Mendocino County, California. A study on this parasite-host system has been underway since 1978 (Schall, 1996; 2002a). Lizards were collected and blood smears made to be stained with Giemsa. Examination under ×1,000 allowed choosing of infected and uninfected lizards for the study; very weak infections, not detectable under the microscope, are rare at Hopland (Perkins et al., 1998).

We trapped sandflies from rodent (*Citellus beecheyi*) burrows; previously fed females are rarely encountered in the traps (Schall, 2002b).

TABLE I. Number of sampled sandflies, ticks, and mites that produced PCR product (band apparent on gel) for a 1,174-bp fragment of the cytochrome b gene of *Plasmodium mexicanum.*

Hours postfeed*	Sandflies†	Ticks†	Mites†
0		8/16	18/18
1	6/6	5/6	1/1
2	8/8	2/4	12/14
5	7/7	0/8	15/16
10	6/6	0/4	16/17
20			1/1
96	6/6		7/15

* Hours since the sandfly or ectoparasite last fed on an infected lizard.
† Number producing bands/total number sampled.

Sandflies and a lizard (either infected or not infected with *P. mexicanum*) were placed into a cloth cage (20-cm cube) and kept at room temperature. Blood-engorged female sandflies were removed and transferred to an 18-ml plastic vial containing a layer of slightly moistened plaster of Paris in its base, and a small piece of fructose-soaked cotton was placed on the screen top of each vial for the insects to feed. We placed the vials into an incubator set at 26 C, the optimal temperature for parasite development (Fialho and Schall, 1995). Sandflies that had fed on an infected lizard were placed into 100% ethanol at 1, 2, 5, 10, or 96 hr postfeed. These sandflies provided a positive control for the method of detecting the parasite. Sandflies that had fed on an uninfected lizard were placed into alcohol at 0 hr postfeed. Lizards naturally infested with mites or ticks were inspected under a dissecting microscope and the ectoparasites were removed to be placed into 18-ml vials with the moist substrate and folded pieces of paper to provide climbing surfaces. The vials were stored at 26 C. Ticks were placed into 100% ethanol from 0, 1, 2, 5, and 10 hr after being removed from the lizard (thus, postfeed), and the mites from 0, 1, 2, 5, 10, 20, and 96 hr. The alcohol in each vial was changed twice in the next 24 hr, and the vials then stored at 4 C.

DNA from the arthropods was extracted using a DNeasy kit (Qiagen, Valencia, California). A single sandfly, mite, or tick was placed into a 1.5-ml vial, allowed to dry for several minutes, and then crushed with a plastic pestle (Sigma, St. Louis, Missouri) in tissue lysis buffer from the kit. The pestle was discarded after a single use. The target 1,174-bp fragment of the *P. mexicanum* mitochondrial cytochrome *b* gene was amplified with the primers DW2 and DW4 (Perkins and Schall, 2002): DW2 forward, 5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′; and DW4 reverse, 5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′. PCR was performed using Ready-to-Go beads (Amersham, Piscataway, New Jersey) in a 25-μl reaction. Added to the bead in a 0.5-ml vial were 22 μl of water, 2 μl of template DNA, and 1 μl of each 10 μM primer. The PCR program used was 94 C for 4 min, followed by 35 cycles of 94 C for 20 sec, 60 C for 20 sec, and 72 C for 90 sec, followed by a final 7-min extension at 72 C. A positive control using template DNA from a known infected lizard's blood was included in each PCR procedure. Two kinds of negative controls were used (i.e., water replacing template DNA and extracted DNA from a sandfly or ectoparasite that had fed on an uninfected lizard). The latter control assured that the primers were amplifying only parasite DNA. PCR product was visualized on 1% agarose gel treated with ethidium bromide. Some PCR products were cleaned through Centricep columns (Princeton Separations, Adelphia, New Jersey) and subjected to cycle sequencing with BigDye terminator mix (ABI, Foster City, California) using the DW2 forward primer, and run on the ABI Prism automated sequencer.

No PCR product (i.e., no band on the gel) was detected for any of the arthropods that had fed on an uninfected lizard, confirming the specificity of the primers for the parasite. PCR product from the sandfly samples always presented a bright band on the gel from 1 to 96 hr postfeed (Table I, Fig. 1A). The PCR product from 2 sandflies, from 10 and 96 hr postfeed, was sequenced; sequences were identical to those reported for *P. mexicanum* (Perkins and Schall, 2002). In contrast, only

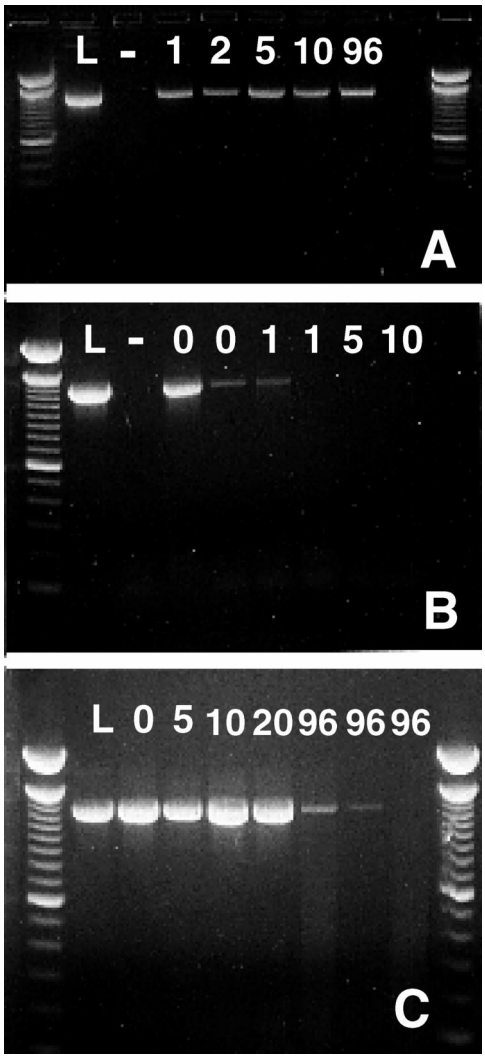

FIGURE 1. Agarose 1% gel stained with ethidium bromide, showing PCR product of an amplified 1,174-bp fragment of the cytochrome *b* gene of *Plasmodium mexicanum.* Parasite DNA was extracted from three blood-feeding arthropods, sandflies (*Lutzomyia* spp.), ticks (*Ixodes pacificus*), and mites (*Geckobiella occidentalis*) that had fed on an infected fence lizard (*Sceloporus occidentalis*). A reference positive control was DNA extracted from the blood of an infected lizard. Negative controls shown are result of PCR using template DNA from a sandfly and tick fed on a uninfected lizard; not shown is the similar negative control from a mite fed on an uninfected lizard included for each PCR run. Hours postfeeding when the ectoparasite was preserved are given (see text). Results for sandflies (**A**), ticks (**B**), and mites (**C**).

half the ticks taken at 0 hr postfeed revealed a positive result, and none were positive by 5 hr postfeed (Table I, Fig. 1B). Of the ticks presenting a positive result, 7 of 15 were faint bands (Fig. 1B), most likely indicating that very little intact template DNA was present in the extraction. A contrasting result emerged for the sampled mites. Fully 94% of 67 mites were positive from 0 to 20 hr postfeed, most with bright bands (only 3 of 67 presented faint bands on the gels). Only half of 15 mites sampled at 96 hr gave a positive result, and all of these bands were faint (Table I, Fig. 1C). PCR product from 2 mites sampled at 10 hr postfeed was sequenced with results identical to the known *P. mexicanum* sequence.

P. mexicanum experiences starkly different fates within each of the blood-feeding arthropods. In sandflies, gametocytes of *P. mexicanum* emerge from their host cell within minutes after the blood leaves the lizard (Osgood and Schall, 2003), undergo mating, and the parasite

reaches maturity on the vector midgut in 6 days at 26 C (Fialho and Schall, 1995). Thus, the parasite survives the digestive processes of the sandfly. Every sandfly tested here revealed a strong positive PCR amplification of the target DNA segment, from 0 to 96 hr after feeding on an infected lizard. These results provide a positive control for the assay method. In ticks, the parasite appears to be killed quickly because the DNA segment was degraded and often failed to amplify even immediately after the feeding tick was removed. In contrast, the long DNA segment was strongly amplified from mites for at least 20 hr after feeding, and half were weakly amplified fully 96 hr after feeding ended.

In ixodid ticks, feeding and digestion is simultaneous, and destruction of the host blood cells occurs immediately after entering the tick, whereas digestion of blood by mites takes longer and requires construction of a peritrophic membrane around the blood meal (Mehlhorn, 2000). Thus, the more leisurely digestion of blood by *G. occidentalis* may allow the parasite to remain alive for at least 20 hr.

The behavior of *G. occidentalis* under natural conditions is unknown, but we have observed fence lizards in cages, and mites very often leave the lizard hosts. Uninfested lizards placed into the cages with lizards carrying the mites are very quickly infested. We have not seen lizards eating the mites, in agreement with observations reported by Klein et al. (1988). However, fence lizards at the Hopland site must often eat mites because *Schellackia occidentalis* infection is common in the lizard population and is transmitted when recently fed mites are ingested by a lizard.

Although a mite passive vector would explain the very fine-scale prevalence patterns of *P. mexicanum* in fence lizards, the lack of digestion of long DNA segments over more than 20 hr in the mite's blood meal, however suggestive, does not demonstrate the parasite cells are alive or able to be transmitted passively if a lizard eats a mite carrying infected blood. We did not expect the results presented here for the mites, and thus did not conduct transmission experiments. Indeed, the purpose of the study was to quickly discount the ectoparasites as possible passive vectors. Therefore, transmission experiments are pending. The mite's digestive tract must be a hostile environment for a *Plasmodium* sp. cell, both because of the presence of lytic enzymes and a steep drop in oxygen concentration. However, sporozoites of *Schellackia occidentalis* survive the gut of the mite and later, consumption by lizards. Avian malaria parasites can survive in blood kept at surprisingly high temperatures within capillary tubes (Caldwall, 1944), and this ability was found in similar experiments with rodent and lizard malaria parasites (Schall, 1990; data not shown), demonstrating that *Plasmodium* sp. parasites are not fragile organisms.

We thank S. M. Osgood for advice and assistance in both the field and laboratory, and E. Glesmann for her help in capturing and maintaining lizards. The staff of the Hopland Research and Extension Center offered significant logistical support, and C. Vaughn allowed us to use a portion of his research laboratory at the field station. The research was funded by a grant from the United States National Science Foundation.

LITERATURE CITED

AYALA, S. C. 1971. Sporogony and experimental transmission of *Plasmodium mexicanum*. Journal of Parasitology **57:** 598–602.

———, AND D. LEE. 1970. Saurian malaria: Development of sporozoites in two species of phlebotomine sandflies. Science **167:** 891–892.

BONORRIS, J. S., AND G. H. BALL. 1955. *Schellackia occidentalis* n. sp., a blood-inhabiting coccidian found in lizards in northern California. Journal of Protozoology **2:** 31–34.

CALDWELL, F. E. 1944. In vitro effects of high temperature on avian malarial parasites. Journal of Infectious Diseases **74:** 189–205.

CHOW-SHAFFER, E., B. SINA, W. A. HAWLEY, J. DEBENEDICTIS, AND T. W. SCOTT. 2000. Laboratory and field evaluation of polymerase chain reaction-based forensic DNA profiling for use in identification of human blood meal sources of *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology **37:** 492–502.

EISEN, R. J., AND N. M. WRIGHT. 2001. Landscape features associated with infection by a malaria parasite (*Plasmodium mexicanum*) and the importance of multiple scale studies. Parasitology **122:** 507–513.

FIALHO, R. F., AND J. J. SCHALL. 1995. Thermal ecology of a malarial parasite and its insect vector: Consequences for the parasite's transmission success. Journal of Animal Ecology **64:** 553–562.

KLEIN, T. A., D. G. YOUNG, E. C. GREINER, S. R. TELFORD JR., AND J. F. BUTLER. 1988. Development and experimental transmission of *Schellackia golvani* and *Schellackia occidentalis* by ingestion of infected blood-feeding arthropods. International Journal for Parasitology **18:** 259–267.

———, ———, S. R. TELFORD, AND R. KIMSEY. 1987. Experimental transmission of *Plasmodium mexicanum* by bites of infected *Lutzomyia vexator* (Diptera: Psychodidae). Journal of the American Mosquito Control Association **3:** 154–164.

LEE, H. J., H. HASSAN, G. HILL, E. W. CUPP, T. B. HIGAZI, C. J. MITCHELL, M. S. GODSEY JR., AND T. R. UNNASCH. 2002. Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. American Journal of Tropical Medicine and Hygiene **66:** 599–604.

MALMQVIST, B., D. STRASEVICIUS, O. HELLGREN, P. H. ALDER, AND S. BENSCH. 2004. Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood. Proceedings of the Royal Society of London (Suppl.) **271:** S152–S155.

MEHLHORN, H. 2000. Encyclopedic reference of parasitology, 2nd edition: Biology, structure, function. Springer-Verlag, New York, New York, 1,252 p.

MUKABANA, W. R., W. TAKKEN, P. SEDA, G. F. KILLEEN, W. A. HAWLEY, AND B. G. J. KNOLS. 2002. Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae). Bulletin of Entomological Research **92:** 233–239.

OSGOOD, S. M., AND J. J. SCHALL. 2003. Gametocyte sex ratio of a malaria parasite: Response to experimental manipulation of parasite clonal diversity. Parasitology **128:** 23–29.

PERKINS, S. L., S. M. OSGOOD, AND J. J. SCHALL. 1998. Use of PCR for detection of subpatent infections of lizard malaria: Implications for epizootiology. Molecular Ecology **7:** 1587–1590.

SCHALL, J. J. 1990. Virulence of lizard malaria: The evolutionary ecology of an ancient parasite-host association. Parasitology **100:** S35–S52.

———. 1996. Malarial parasites of lizards: Diversity and ecology. Advances in Parasitology **37:** 255–333.

———. 2002a. Parasite virulence. *In* The behavioural ecology of parasites, E. E. Lewis, J. F. Cambell, and M. V. K. Sukhdeo (eds.). CABI Publishing, Oxon, U.K., p. 293–313.

———. 2002b. Gametocyte sex ratio of a malaria parasite: Experimental test of heritability. Journal of Parasitology **88:** 494–498.

———, AND A. B. MARGHOOB. 1995. Prevalence of a malarial parasite over time and space: *Plasmodium mexicanum* in its vertebrate host, the western fence lizard, *Sceloporus occidentalis*. Journal of Animal Ecology **64:** 177–185.

———, H. R. PRENDEVILLE, AND K. HANLEY. 2000. Prevalence of the tick, *Ixodes pacificus*, on western fence lizards, *Sceloporus occidentalis*: Trends by site, gender, size, season, and mite infestation. Journal of Herpetology **34:** 160–163.

TALLEKLINT-EISEN, L., AND R. S. LANE. 1999. Spatial and temporal variation in the density of *Ixodes pacificus* (Acari: Ixodidae) nymphs. Environmental Entomology **29:** 272–280.