RESEARCH ARTICLE

PARASITOLOGY

Hidden in plain sight: Cryptic and endemic malaria parasites in North American white-tailed deer (*Odocoileus virginianus*)

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Malaria parasites of the genus *Plasmodium* are diverse in mammal hosts, infecting five mammalian orders in the Old World, but were long considered absent from the diverse deer family (Cervidae) and from New World mammals. There was a description of a *Plasmodium* parasite infecting a single splenectomized white-tailed deer (WTD; *Odocoileus virginianus*) in 1967 but none have been reported since, which has proven a challenge to our understanding of malaria parasite biogeography. Using both microscopy and polymerase chain reaction, we screened a large sample of native and captive ungulate species from across the United States for malaria parasites. We found a surprisingly high prevalence (up to 25%) and extremely low parasitemia of *Plasmodium* parasites in WTD throughout the eastern United States. We did not detect infections in the other ungulate species nor in western WTD. We also isolated the parasites from the mosquito *Anopheles punctipennis*. Morphologically, the parasites resemble the parasite described in 1967, *Plasmodium odocoilei*. Our analysis of the cytochrome b gene revealed two divergent *Plasmodium* clades in WTD representative of species that likely diverged 2.3 to 6 million years ago, concurrent with the arrival of the WTD ancestor into North America across Beringia. Multigene phylogenetic analysis placed these clades within the larger malaria parasite clade. We document *Plasmodium* parasites to be common in WTD, endemic to the New World, and as the only known malaria parasites from deer (Cervidae). These findings reshape our knowledge of the phylogeography of the malaria parasites and suggest that other mammal taxa may harbor infection by endemic and occult malaria parasites.

INTRODUCTION

Malaria parasites exploit a diverse array of vertebrate hosts (squamate reptiles, birds, and mammals) and dipteran insect vectors (mosquitoes, midges, black flies, among many others) from every terrestrial habitat and every continent except Antarctica (*1*). The *Plasmodium* species of avian hosts have been described from most bird orders and families and are geographically cosmopolitan with some species even reaching oceanic islands and displaying a worldwide distribution (*2*). The *Plasmodium* species that infect lizards are likewise broadly distributed, also occurring on oceanic islands and large isolated landmasses such as New Zealand (*3*). Birds are highly mobile and thus can carry parasites over the continents, whereas the distribution of *Plasmodium* in squamate reptiles may represent repeated lateral transfer between birds and lizards

over their evolutionary history (*4*). In contrast, the *Plasmodium* species of mammals have a more limited distribution, long thought to be restricted to the Old World where *Plasmodium* species are known from five mammal orders, namely, Primates (lemurs, monkeys, and apes, including five nominate species in humans), Rodentia (thicket rats, porcupines, and flying squirrels), Chiroptera (bats), Dermoptera (colugos), and Artiodactyla [mouse deer (Tragulidae), antelope, and Asian water buffalo (Bovidae)] (*3*, *5*). Surveys showed *Plasmodium* parasites to be absent in the diverse deer family Cervidae, which is one of the largest and most widely distributed mammalian families. In addition, no endemic *Plasmodium* parasite was known from any mammal in the New World. Two species of *Plasmodium* described from numerous species of South American monkeys are now known to be lateral transfers of two human parasites, *Plasmodium vivax* and *Plasmodium malariae*, probably since European contact (*6*). Thus, *Plasmodium* parasites have been described from diverse mammal taxa and over a large geographic range including Africa, mainland Asia, and the large islands of Southeast Asia, but not from the New World.

These perplexing patterns, the apparent absence of *Plasmodium* parasites from mammals in the New World and their absence from the diverse deer family, were challenged by the 1967 report of a new malaria parasite, later described in 1980 as *Plasmodium odocoilei*, from a single white-tailed deer (WTD) *Odocoileus virginianus*, from Texas, USA (*7*). This animal had been splenectomized in an attempt to reveal occult or hidden infections, and even then, prevalence within the blood of the new parasite was extremely low (one parasite per 30,000 red blood cells) (*8*). Only a single blood smear from the infection was preserved with this smear now deposited at the Natural History Museum in London, UK (*9*). Despite many studies of blood parasites of WTD,

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a common big game species in North America, over the past half century, *P. odocoilei* has not been observed again (*8, 10, 11*). Many spurious reports of *Plasmodium* species have entered the parasitological literature, so the WTD parasite may well have been misidentified. However, the illustrations in the species description resemble *Plasmodium*, including the presence of both multinucleated schizont stages and hemozoin pigment, and one author, P. C. C. Garnham, was one of the field's eminent scholars and would have been unlikely to misidentify the parasite.

During a polymerase chain reaction (PCR)–based survey for the vectors of avian malaria parasites at the Smithsonian's National Zoological Park (NZP), we recovered an enigmatic malaria parasite sequence. By phylogenetic analysis of the parasite's cytochrome b gene (*cytb*), this sequence placed within the overall *Plasmodium* clade but was divergent from other sequenced *Plasmodium* taxa. WTD was identified as the blood meal source by analysis of the vertebrate DNA (*cytb*) isolated from the blood meal of one of the *Plasmodium*-positive mosquitoes. This finding led us to conduct a major survey of WTD and other wild native and zoo ungulate species from across the United States and to investigate the possible origins of malaria parasites in New World mammals. We report the rediscovery of *Plasmodium* parasites in WTD

and further find that these malaria parasites, which have remained hidden for so long in North America's most popular game species, the WTD, are common, diverse, and endemic.

RESULTS

Vector and vertebrate host sampling

A total of 1978 individual mosquitoes of 27 species were sampled at two sites, NZP, Washington, DC, and San Diego County, CA (Fig. 1 and table S1). Salivary glands were dissected out of each mosquito and screened by nested PCR for *Plasmodium* infection using primers that target a fragment of the *cytb* gene on the parasite's mitochondrial genome (*4*). A phylogenetic analysis of the sequences obtained from the salivary glands of *Anopheles punctipennis* (2 of 35, prevalence of 5%) placed the parasite within the overall *Plasmodium* clade but not close to other sequenced mammalian *Plasmodium* taxa. All other parasite sequences retrieved from mosquito salivary glands placed within the avian *Plasmodium* clade. WTD was identified as the blood meal source from the blood meal of one of the *Plasmodium*-positive *A. punctipennis*. This native mosquito
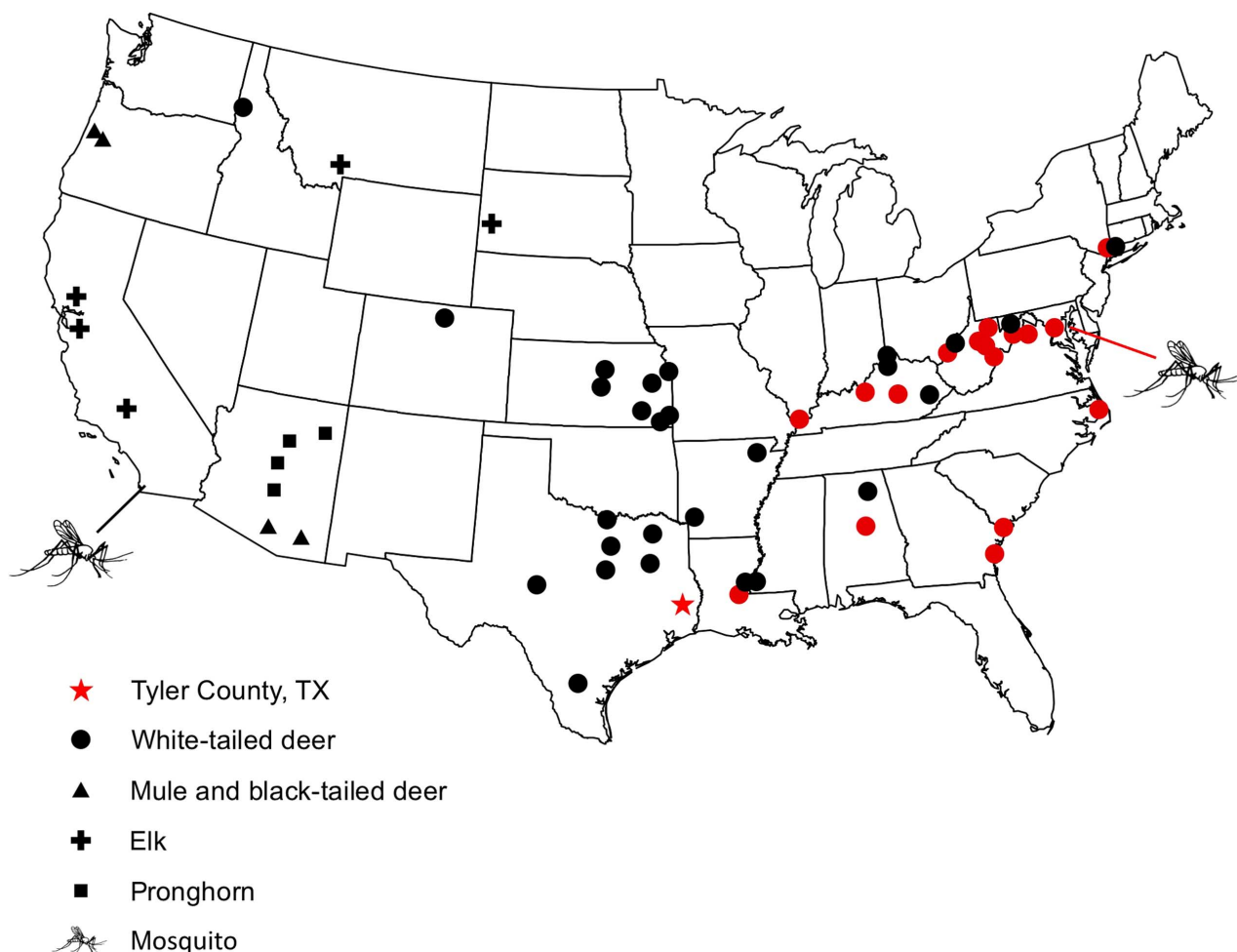
Fig. 1. Sampling sites of mosquitoes and wild ungulate hosts for *Plasmodium* parasites. A red star denotes the original sampling location for *P. odocoilei* from a single, splenectomized WTD in Tyler County, TX. Sites from which positive infections were discovered are indicated in red.

species occurs in more mesic habitats throughout North America, taking blood meals from large mammals (*12*), and historically was a vector of introduced human malaria parasites in North America (*13*).

We surveyed WTD across their range as well as two other native cervid species that overlap with the range of the WTD, the mule deer (*Odocoileus hemionus*, the closest sister taxon to WTD) and elk (*Cervus canadensis*). We also sampled pronghorn (*Antilocapra americana*), a distant artiodactylid relative of deer, from parts of their range that overlap with WTD (Fig. 1 and table S2). Because the WTD parasite could have been acquired from an exotic or domestic host, nine captive species of ungulates from NZP campuses in Washington, DC, and Front Royal, VA, were screened for *Plasmodium* (table S3). All host species were screened for *Plasmodium* infection by nested PCR of blood or tissue samples. Microscopic examination of blood smears was then carried out for a subset of the samples for confirmation of infection and visualization of parasite morphology.

Parasite screening

The nested PCR-based screening of 308 WTD from 17 states found 41 infected animals from 17 counties throughout 10 states in the eastern and southern United States (Fig. 1 and table S2). Overall prevalence at the *Plasmodium*-positive sites was 18% but reached approximately 25% at sites in Virginia and West Virginia. None of the other sampled native ungulate species (a total of 151 individuals of three species) or exotic or domestic ungulate species (39 individuals of nine species) were found to harbor *Plasmodium* infection (tables S2 and S3). A total of four animals produced double-nucleotide peaks on sequence electropherograms indicative of mixed infections (*14, 15*). Ambiguities were only present at one or two nucleotide positions, and each of these mixed infections could be unequivocally assigned to single parasite infections present in other infected WTD.

Examination of blood smears from infected WTD revealed parasites that were diagnosed as *Plasmodium* by morphology including the diagnostic characters of hemozoin pigment and asexual reproduction in blood cells, and these WTD parasites closely matched the illustrations in the original species description of *P. odocoilei* (*8*). Examination of the original type smear [loaned from the Natural History Museum, London, UK (specimen number 742 H of the Garnham collection)] found parasites with similar morphology to those from the newly sampled WTD (Fig. 2), including grossly enlarged infected red blood cells, localized pigment, spiked projections of the gametocytes, and conspicuous vacuoles. When compared to other described mammalian *Plasmodium* species,

the morphology of the WTD parasite was most similar to *Plasmodium cephalophi* of the grey duiker (*Sylvicapra grimmia*), an African antelope (*3*). No blood samples had been stored from the species description, and the original blood smear could not be destructively sampled for molecular analysis.

Molecular analyses

To determine where the WTD parasites place within a broad phylogeny, four genes from the parasites' three genomes were amplified and sequenced using primers specific for haemosporidian parasites: *cytb* and cytochrome oxidase I (*coI*) from the mitochondrial genome, adenylosuccinate lyase (*asl*) from the nuclear genome, and caseinolytic protease (*clpC*) from the plastid genome (*4*). Haemosporidians, like most parasites from the phylum Apicomplexa, carry a unique organelle, the apicoplast, with its own genome. Bayesian phylogenetic analyses placed the WTD parasites not closely related to *Plasmodium* of other mammal hosts but rather as sister to *Polychromophilus* parasites of bats from Eurasia and Africa (Fig. 3). The genus *Plasmodium* was recovered as polyphyletic in the resulting topology with parasites identified to this genus recovered in three separate clades: *P. odocoilei* and *Polychromophilus*, the *Plasmodium* species of avian and squamate hosts plus *Nycteria* parasites of Old World bats, and a paraphyletic clade of primate and rodent *Plasmodium* species plus *Hepatocystis* parasites of Old World bats (Fig. 3). Thus, the clade of *Plasmodium* parasites of WTD in North America place most closely related to malaria parasites of Old World bats.

The *cytb* gene is often used in studies of species-level diversity of malaria parasites, and a phylogenetic analysis of sequence data from this gene revealed a number of parasite haplotypes isolated from WTD (Fig. 4) (*16*). Two main clades representative of species differed by 3% of 614 base pairs (bp). Individual phylogenetic analysis of each of the other three genes individually (*coI*, *clpC*, and *asl*) also corroborate reproductive isolation of these two main clades and their status as distinct species. Using two independent molecular clock estimates for divergence at the mitochondrial *cytb* gene for malaria parasites (*17, 18*), we found that these two clades likely diverged between 2.3 and 6 million years ago (Ma). A recent study evaluating divergence times for mammalian *Plasmodium* taxa based on analysis of thousands of nuclear genes (*19*) confirms mitochondrial time estimates for very recent divergences, which would include those estimated for the two WTD *Plasmodium* species in our study. The ancestor of the WTD is estimated to have traveled across the Bering Land Bridge into North America in the Miocene 4.2 to 5.7 Ma (*20*). Diversity of *cytb* haplotypes within one
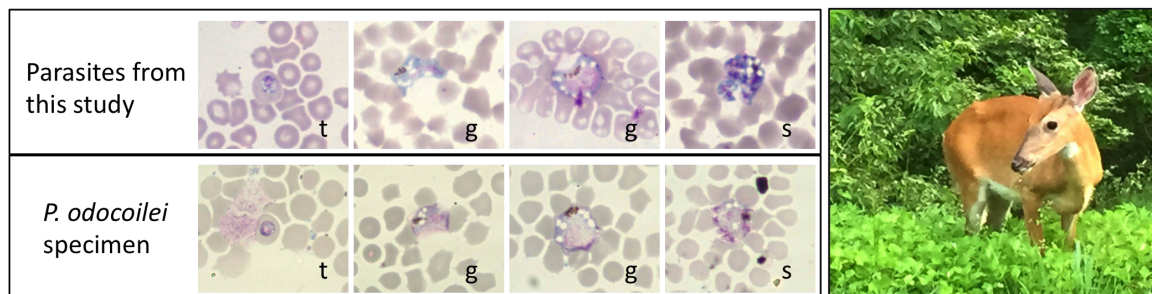
Fig. 2. Malaria parasites of the WTD as viewed under the light microscope. Microphotographs of parasites identified as *P. odocoilei* of the WTD including blood smears prepared from our study and the original type specimen (Natural History Museum, London, UK) for the species. Shown are trophozoites (t), gametocytes (g), and schizonts (s). Also shown is a picture of a WTD from one of the high-prevalence sites in the study.
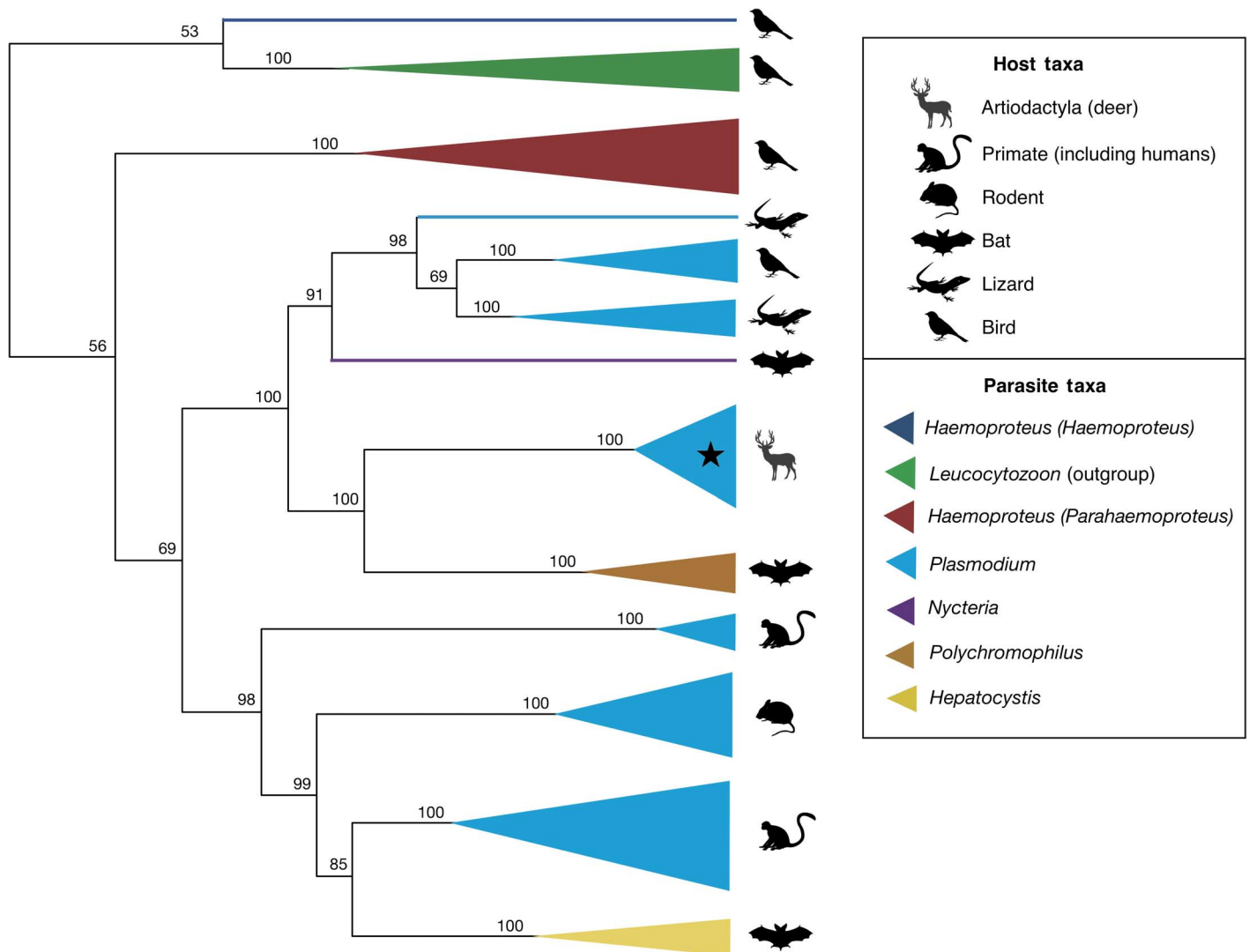
Fig. 3. A multigene phylogeny displaying the relationships between *Plasmodium* lineages from WTD (clade denoted by black star) and other malaria parasites of vertebrate hosts. Host taxa are indicated by different symbols and parasite genera by different colors, and size of triangles indicates relative number of parasite taxa contained within a clade. The phylogeny was reconstructed by partitioned Bayesian analyses of mitochondrial (*cytb, co1*), apicoplast (*clpC*), and nuclear (*asl*) genes and rooted with *Leucocytozoon* taxa. Bayesian posterior probability values are indicated above nodes.

*Plasmodium* clade (clade 2; Fig. 4) is about 0.5%, estimated with an origin at 0.4 to 1.0 Ma.

DISCUSSION

We found *Plasmodium* parasites that are widespread and locally common in WTD, the iconic large game animal of North America. This conclusion is supported by both microscopy of parasite morphology and phylogenetic analysis of gene sequence data. We also isolated *Plasmodium* DNA from the salivary glands of *A. punctipennis*, suggesting that this mosquito species is the vector host of the WTD parasites. The parasites clearly concord with the morphology and life history characters that diagnose the genus *Plasmodium* including vertebrate and dipteran hosts, asexual replication in the blood cells by schizogony, production of male and female gametocytes in the blood cells, and visible hemozoin pigment within the schizonts (meronts) and gametocytes. The parasite

matches the morphology of *P. odocoilei* described five decades ago from a single animal but not observed since. Furthermore, phylogenetic analysis of genes from all three genomes of the parasites placed the WTD parasites within the overall *Plasmodium* clade. Together, our microscopy and molecular results of both vertebrate and vector hosts corroborate the rediscovery of malaria parasites from the WTD in North America. The malaria parasites, which we regard as the *P. odocoilei* species group, are confirmed as the only known endemic *Plasmodium* of a New World mammal and the only *Plasmodium* known from a deer.

The presence of multiple *Plasmodium* clades in the WTD argues for a long evolutionary history of *Plasmodium* parasites and their WTD hosts extending back, perhaps, to the origin of WTD in North America. A few lines of evidence support the passage of these WTD parasites over the Bering Land Bridge within the ancestor of WTD rather than host shifts into WTD from introduced ungulates such as captive animals in zoos. First, at least two likely species of *Plasmodium*
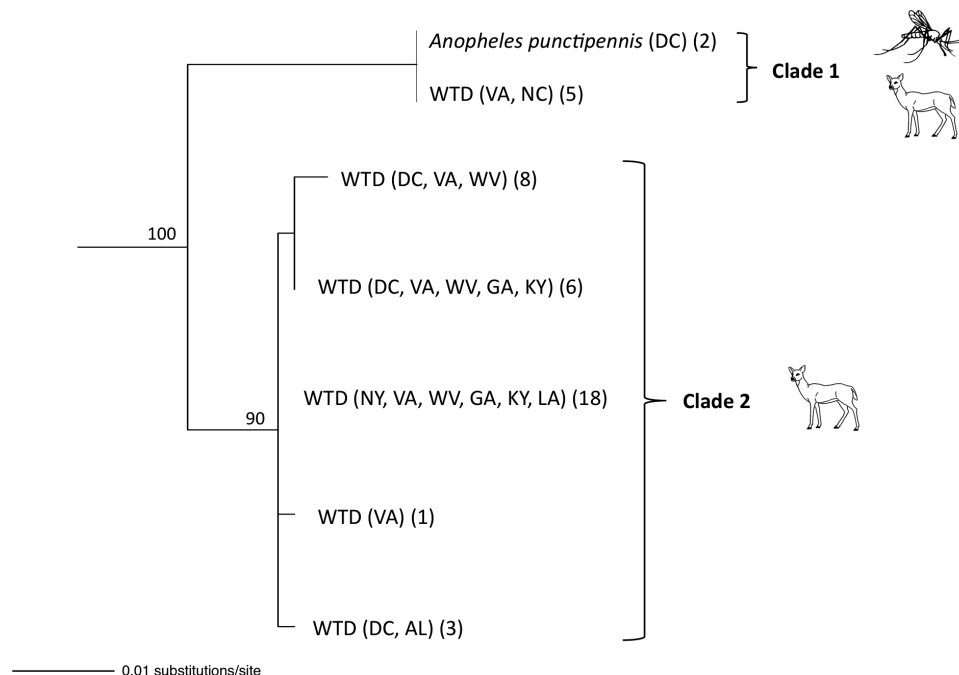
Fig. 4. Evolutionary relationships of *Plasmodium* parasites isolated from WTD and mosquitoes (*A. punctipennis*) in our study. The states from which parasites were sampled and sample sizes are indicated (state abbreviations included in table S2). The phylogeny and bootstrap support values (displayed above nodes) were estimated by maximum likelihood analysis of 614 bp of the *cytb* gene. Clades 1 and 2 are approximately 3% divergent.

parasites were isolated from WTD (based on their genetic divergence), and these parasites are closely related. Within one of the WTD clades (lineage 2; Fig. 4), we found additional variation (up to 0.5%) that may be indicative of substantial intraspecific variation. The application of multiple independent molecular clock estimates for the malaria parasites (*17–19*) supports the divergence of these parasite clades to be roughly concordant with the passage of the WTD ancestor through Beringia. In addition, we failed to detect parasite DNA in a sample of nonnative ungulate species in the United States, including domestic species and species housed in collections at zoological parks. *Plasmodium* parasites have been described from the Asian mouse deer (Tragulidae) and the antelope and Asian water buffalo (Bovidae) in Africa, all of which have been introduced into the New World, but it is unlikely that parasites from these species would jump numerous times into deer and thus into a new host family (Cervidae) (*8*). Clearly, more research including sampling of Old World ungulate hosts, both native and introduced, and genetic characterization of their *Plasmodium* parasites are needed to further support or refute hypotheses on the origin of malaria parasites in New World deer. If the parasites did indeed travel over the Bering Land Bridge with a herd of ancestral North American deer, it suggests that suitable temperatures existed in the high arctic for successful transmission of *Plasmodium* parasites by *Anopheline* mosquitoes. This potential scenario highlights the importance of the Bering Land Bridge and interglacial periods in the passage of vector-borne pathogens into the New World.

WTD may have carried multiple parasite lineages from their ancestor population during the migration across Beringia (*20*), or Pleistocene glaciations forced WTD into ice-free refugia, as in the mule deer *O. hemionus* (*21, 22*), leading to the diversification of both the WTD and parasites. WTD were nearly extirpated throughout much of the

eastern and southern portions of their range due to overhunting, exploitation, and other causes, with reintroductions into these areas in the 1920s to the 1970s (*23*). Thus, it is likely that any biogeographic signals of the parasites would have been lost. In summary, the absence of parasites in the exotic host species we sampled and the finding of multiple closely related *Plasmodium* lineages within the WTD support the endemic nature of these WTD malaria parasites in North America.

Our results beg the question of why diverse malaria parasites that are specialized to WTD remained cryptic in a large game mammal that has long been the center of study for wildlife biologists and managers. Most likely, the answer is the occult nature of all of the infections observed for *P. odocoilei*, with extremely low parasite density (an estimated ~1/65,000 red blood cells infected). This is not unusual for the *Plasmodium* infecting mammals including the malaria parasites of humans. Examples include *P. cephalophi* and *Plasmodium brucei* in the common duiker (*S. grimmia*), an African antelope, *Plasmodium traguli* from the chevrotain (*Tragulus javanicus*) of Southeast Asia, *Plasmodium inui* in Asian macaque monkeys, and *Plasmodium girardi* in lemurs (*3*). Some of these species were discovered only from hosts that had been splenectomized as an experimental method to reveal low-level blood infections. Very-low-level infections, although not as extreme, are also common in many avian malaria parasites (*2, 24*). This life history trait, of producing few parasites in the blood of the vertebrate host, is clearly sufficient for the successful transfer to the insect host and shows that the gametes produced by the parasite are able to find what are likely rare mates in the vector blood meal. When feeding, *Anopheles* take approximately 3 µl of blood, and WTD blood contains approximately 8×10^6 erythrocytes/µl; a blood meal would therefore contain hundreds of parasite cells, even for such occult infections (*25, 26*). Together, these findings suggest that for some species of *Plasmodium*, low-level infections are successful in transmission.

Our findings highlight the importance of molecular methods in parasite discovery. Through the application of sensitive PCR-based screening, we detected low parasitemia infections in WTD previously undetected under the light microscope for a half century. Phylogenetic methods also allowed for the identification of multiple cryptic *Plasmodium* species in WTD. The discovery of *Plasmodium* parasites that are common and widespread in such a well-studied game species as the WTD, yet not recognized for so long, argues that other malaria parasites of mammals are yet to be discovered, perhaps in surprising host species within plain sight. The coupling of light microscopy and molecular methods and increased surveillance for mammalian *Plasmodium* parasites promises to further expand our knowledge of the systematic diversity and phylogeography of the malaria parasites.

MATERIALS AND METHODS

Experimental design
The main objectives of our study were to examine the distribution of the *Plasmodium* parasites of New World mammals including their vector and vertebrate hosts, geographic range, and possible origin in the New World. We sampled hundreds of vertebrate hosts from across the continental United States, including both wild and native ungulate species as well as nonnative and domestic ungulate species. Samples were screened by PCR, which allowed for detection of low-level infections, and additionally by microscopy for the WTD samples, which allowed for visualization of the parasites and confirmation of development in the vertebrate host. Thousands of mosquitoes were screened by PCR for malaria parasites as well. Analysis of sequence data from the parasites then allowed us to investigate the diversity of parasites, their phylogenetic relationships with other malaria parasites, and to estimate divergence dates.

Sampling of vertebrate hosts
WTD samples were obtained during necropsy of dead animals, health assessments, or other research purposes by the Smithsonian Conservation Biology Institute, the Southeastern Cooperative Wildlife Disease Study, and the American Museum of Natural History. We screened a total of 308 WTD samples from 25 counties in 17 states across the range of the species in the United States (table S2 and Fig. 1). Elk were sampled from five counties in three states, mule deer from four counties in two states, and pronghorn from four counties in one state (table S2 and Fig. 1). Mule deer, elk, and pronghorn samples were obtained from areas that overlap with the geographic range of the WTD. A total of 39 collection animals of nine species were screened from the NZP campuses in Washington, DC, and Front Royal, VA, including two antelope species and two deer species (table S3). Samples screened in this study include spleen and liver tissue and blood collected during the warm months in which biting mosquitoes are active (May through October). DNA extractions were obtained from heart and ear tissues for the Connecticut and New York samples. All sampling methods were approved by the Institutional Animal Care and Use Committee.

As part of other research studies, WTD were live caught at the Front Royal Campus of the NZP, which enabled zoo veterinary staff the opportunity to prepare blood smears from the animals in addition to blood collection for PCR screening. Blood was collected from the jugular vein into heparinized syringes, and thin blood smears were immediately prepared in the field. Blood smears were then fixed in methanol, stained with Giemsa, and examined for blood parasites under ×100 magnification.

Sampling of invertebrate hosts
Mosquitoes were sampled at the NZP in Washington, DC, from mid April through late October and from sites throughout San Diego County in California in April and May using Centers for Disease Control (CDC) miniature light traps baited with CO_2 and gravid traps baited with rabbit pellet–infused water. Male mosquitoes were discarded, while blood-fed, unfed, and gravid females were separated from each other. Approximately 20% of gravid females from each mosquito species and all blood-fed individuals were then screened for malaria parasite infection by nested PCR (table S1). Mosquitoes were determined to be gravid by dissection of ovaries under the dissection scope and visual inspection of ovaries on glass microscope slides under the light microscope at 10×. All blood-fed and selected parous mosquitoes were identified to species based on morphology with further differentiation of *Culex* species carried out by PCR for infected individuals (*27*). The salivary glands of each mosquito were then carefully extracted under a dissection scope using sterile instruments, and DNA was extracted using the Qiagen BioSprint 96 System following the manufacturer's guidelines. The abdomen from each blood-fed individual was removed using sterile instruments and transferred to a microcentrifuge tube with buffer ATL where it was agitated with a sterile plastic pestle before DNA extraction using the Qiagen BioSprint 96 System following the manufacturer's instructions.

Parasite screening and phylogenetic analysis
DNA was extracted from blood or tissues using a Qiagen DNeasy Blood and Tissue Kit or the Qiagen BioSprint 96 System following the manufacturer's guidelines. A 614-bp fragment of the *cytb* gene in malaria parasites was amplified by nested PCR using the conserved primers DW2/DW4 followed by DW1/DW3 (*28*). Negative controls were included for each round of PCRs, and no contamination was detected. PCR products were visualized by gel electrophoresis, purified using ExoSAP-IT (Affymetrix), and cycle-sequenced using BigDye Terminator v3.1, and the sequencing products were cleaned with Sephadex G-50 columns and then sequenced on an ABI 3130*xl* Sequencer. Sequences were edited and aligned using Sequencher version 5.0 (Gene Codes). Using Geneious, maximum likelihood methods were used to reconstruct a phylogeny and estimate nodal support values for the *cytb* data set.

For the four-gene phylogeny reconstruction, DNA from four genes across three genomes was amplified and sequenced, including *cytb* and *coI* from the mitochondrial genome, *clpC* from the plastid genome, and *asl* from the nuclear genome (*4*). All sequences were edited and aligned by eye using Sequencher v5.0. BEAST v1.8.0 (*29*) was used to generate a Bayesian tree and posterior probability values. Mitochondrial (*cytb* and *coxI*) data were partitioned into first plus second and third codon positions, where *clpC* and *asl* data were not, as determined by PartitionFinder (*30*). A GTR + I + G model was selected with equal nucleotide frequencies and estimated substitution rate matrix. The starting tree topology was obtained via the graphical user interface implementation of RAxML (*31*), and a Yule speciation process was applied. An uncorrelated relaxed clock (*32*) was used, and a Markov chain Monte Carlo with chain length of 50,000,000 was run. The first 25% of trees were discarded as burn-in, and the tree was summarized with TreeAnnotator (*33*).

Extraction products from the blooded mosquito abdomens were used to amplify a short fragment of the vertebrate *cytb* gene using primers SteeriF1/SteeriR1 (*34*). PCR products were visualized on agarose gels, cleaned using ExoSAP-IT, and sequenced as described above.

Using the BLAST function in the GenBank and BOLD databases, blood meal origin was determined.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/2/e1501486/DC1

Table S1. Mosquito species screened by nested PCR for *Plasmodium* infection for the study.
Table S2. County and state locations for the wild ungulate species sampled in our study and proportion found to be infected with malaria parasites at each site by nested PCR.
Table S3. Collection animals screened at the NZP's campuses in Washington, DC, and Front Royal, VA, for malaria parasite infection by nested PCR.
Table S4. GenBank accession numbers for *Plasmodium* sequences generated from WTD and *A. punctipennis* and used in the phylogenetic analyses in this study.

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Hidden in plain sight: Cryptic and endemic malaria parasites in North American white-tailed deer (Odocoileus virginianus)

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