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VERTEBRATE HOSTS AND PHYLOGENETIC RELATIONSHIPS OF AMPHIBIAN TRYPANOSOMES FROM A POTENTIAL INVERTEBRATE VECTOR, *CULEX TERRITANS* WALKER (DIPTERA: CULICIDAE)

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ABSTRACT: The blood meals of field-collected female *Culex territans* (Diptera: Culicidae) were concurrently assayed for the presence of trypanosomes and for vertebrate host identification. We amplified vertebrate DNA in 42 of 119 females and made positive identification to the host species level in 29 of those samples. Of the 119 field-collected *Cx. territans* females, 24 were infected with trypanosomes. Phylogenetic analysis placed the trypanosomes in the amphibian portion of the aquatic clade of the Trypanosomatidae. These trypanosomes were isolated from *Cx. territans* females that had fed on the frog species *Rana clamitans*, *R. catesbeiana*, *R. virgatipes*, and *Rana* spp. Results support a potential new lineage of dipteran-transmitted amphibian trypanosomes may occur within the aquatic clade. The frequency in which female *Cx. territans* acquire trypanosomes, through diverse feeding habits, indicates a new relationship between amphibian trypanosomes and mosquitoes that has not been examined previously. Combining *Trypanosoma* species, invertebrate, and vertebrate hosts to existing phylogenies can elucidate trypanosome and host relationships.

Culex territans Walker (Diptera: Culicidae) is found throughout most of the Northern Hemisphere (Knight and Stone, 1977). Females prefer amphibian blood meals (63%), feeding intermittently on Reptilia, Aves, and Mammalia (Savage et al., 2007). In New Jersey, *Cx. territans* occasionally feed on reptilian and avian sources, but they prefer amphibians (88.5%) (Crans, 1970). *Culex territans* is a competent vector for several amphibian parasites, including the nematode *Foleyella flexicauda* (Benach, 1971). In New Jersey, bullfrogs (*Rana catesbeiana*) show high levels of co-infection with *F. flexicauda* and trypanosomes (Benach, 1971). Amphibian trypanosomes infect most anuran species (Bardsley and Harmsen, 1973). Barta and Desser (1984) found trypanosomes are prevalent parasites infecting amphibians in Ontario, Canada, an area where *Cx. territans* is common (Desser et al., 1973).

Trypanosomes are cosmopolitan vertebrate parasites that are transmitted by invertebrate vectors (Hamilton et al., 2004). Within *Trypanosoma* spp., the biology, hosts, and mode of transmission are unknown for many species. Phylogenetic analysis is used to determine evolutionary origins and relationships of trypanosomes to clarify fundamental questions on the biology of these species. Various Trypanosomatidae phylogenies show coevolution of trypanosomes with vertebrate hosts, invertebrate vectors, and biogeography. In most interpretations, the amphibian trypanosomes were among the earliest to diverge from monoxenous trypanosomatids (Hamilton et al., 2004). Amphibian trypanosomes are placed within a monophyletic group referred to as the aquatic clade (Hamilton et al., 2007), which are primarily leech transmitted. Trypanosomes within this clade also occur in terrestrial vertebrates, suggesting an insect vector might transmit trypanosomes within the aquatic clade (Barta and Desser, 1984). A further understanding of vertebrate and invertebrate hosts of aquatic trypanosomes may shed light on unresolved phylogenies, and aid in understanding the evolution of parasitism within this group.

It has been proposed that *Cx. territans* might serve as a vector for amphibian trypanosomes, including *T. ranarum* (Barta and Desser, 1984). Transmission of amphibian trypanosomes has been demonstrated in nematoceran Diptera, including Corethrellidae (Johnson et al. 1993), and Psychodidae (Anderson, 1968), indicating that leeches are not the only vectors of amphibian trypanosomes. Van Dyken et al. (2006) detected trypanosomes in unengorged *Cx. pipiens* and *Cx. tarsalis*, and they suggested that trypanosomes might increase the vector competence of West Nile virus.

Parasitic trypanosomes rely on blood-feeding vectors for transmission to new hosts (Hamilton et al., 2007). Trypanosome transmission occurs during blood feeding via saliva, by fecal deposits being rubbed into wounds, or by host ingestion. Desser et al. (1973) found *T. rotatorium* development to the epimastigote stage in *Cx. territans*, but they were unable to experimentally infect *R. pipiens* with epimastigotes (Desser et al., 1975). The authors did not attempt transmission via blood feeding, or with other species of trypanosomes. Trypanosomes require suitable host conditions, such as a specific pH, to initiate the development into the infective stage (Ucros et al., 1983); thus, a species might require blood feeding to initiate development. Martin and Desser (1991) found that infective stage *T. fallisi*, which infects amphibians, migrated to the leech's proboscis during blood feeding.

Our goal was to further the understanding of amphibian trypanosomes and phylogenies, by examining the life history of a potential invertebrate vector, *Cx. territans*. The objective was to identify the vertebrate sources of blood meals, while concurrently examining *Cx. territans* females for trypanosomes. Adding a tritrophic study of trypanosome species, vertebrate, and invertebrate hosts to existing phylogenies can elucidate the evolution of parasitism within the Trypanosomatidae. Our hypothesis was that *Cx. territans* feeds predominantly on amphibian blood, and acquires amphibian trypanosomes during blood feeding.

MATERIALS AND METHODS

Mosquito collections

Culex territans females were collected in New Jersey using resting box, light, and carbon dioxide-baited traps, from 2003 to 2007, through a statewide vector surveillance program (Crans and McCuiston, 1993). Resting boxes were set out each year from May to October in Atlantic, Burlington, Camden, Cape May, Monmouth, and Salem counties. In 2005 and 2006, resting boxes were also set in early April in Bergen and Sussex counties. Mosquitoes were anesthetized using triethylamine, hand aspirated, and placed in 10-ml glass vials within clear

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TABLE I. Primers used to amplify regions of target DNA from engorged *Culex territans* field-collected in New Jersey, 2003–2007.

Target region	Forward and reverse sequences	Amplicon size (bp)
Amphibian Cyt b	THC TNT CNG CHG CCC CVT A GAG CGD AGR ATN GCR TAR GC	402
Herptile Cyt b	GGN TCR TCC AAC CCA AYW G TTT DGC DAD DGG DCG RAA N	518
Vertebrate Cyt b	TGA GGA CAA ATA TCA TTY TGA GG AGT TTT CTG GGT CTC CTA	358
Trypanosoma gGAPDH	GTG CAY GGC AAG TTC AAG TA GTA CGA GTG GAT CGT CGT CA	426

plastic bags in a cooler with dry ice. Blooded specimens of *Cx. territans* were identified to species on a chill table and placed in an individually labeled 1.5-ml microcentrifuge tube. Trap type and location, Sella stage of blood meal digestion (WHO, 1975), and date were recorded. Specimens were stored at −70 C.

Primer design

Unique primers were designed using Primer3 software (Rozen and Skaletsky, 2000). Random sequences were chosen for trypanosomes and amphibians from GenBank and aligned using BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999) to create a consensus sequence. For development of the trypanosome primers, the sequences included representatives of both the terrestrial and amphibian clades. For the amphibian primers, all amphibians found in New Jersey, with representative sequences found in GenBank were used in the primer development. Primers were selected based on melting temperature, GC content, primer dimers, and hairpins. New primers were developed to ensure that mosquito DNA was not going to be amplified. Mosquito sequences were added to the alignments to exclude amplification of mosquito DNA with the developed primers. Two primer sets were developed (Table I), 1 set to amplify a 406-base pair (bp) region of the cytochrome b (Cyt b) gene in amphibians and 1 set to amplify a 426-bp region of the glycosomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of trypanosomes. The GAPDH gene was chosen due to its slow rate of molecular evolution and availability of sequences (Hamilton et al., 2004). Primers for determining cytochrome b in mammal, bird, and reptile DNA were based on previous studies (Scott, 2003). Primers amplifying host DNA were tested on *Rana catesbeiana* (bullfrog), *R. clamitans* (green frog), *Bufo americanus* (American toad), *R. sphenocephala* (southern leopard frog), *R. virgatipes* (carpenter frog), *Chrysemys picta* (painted turtle), *Macrochelys temminckii* (snapping turtle), *Nerodia sipedon* (water snake), *Gallus gallus* (chicken), *Corvus brachyrhynchos* (American crow), *Equus caballus* (horse), *Procyon lotor* (raccoon), and *Sylvilagus floridanus* (eastern cottontail rabbit) DNA.

Molecular techniques

Mosquitoes were pinned to a Styrofoam block, and the legs and wings were removed to minimize extraneous DNA. The mosquito's abdomen was cut from the thorax, and the blood packet was rolled out using a probe. Blood packets were immediately transferred to a sterile 1.5-ml microcentrifuge tube at −70 C until further processing.

Mosquitoes were homogenized in 140 μl of DNAzol® (Molecular Research Center, Cincinnati, Ohio) using a sterile pestle, and then they were placed in a heat block at 95 C for 10 min. Tubes were centrifuged at 11,000 rpm for 10 min, the supernatant was removed, and 50 μl of 100% ethanol was added to each tube. Samples were centrifuged twice for 10 min at 11,000 rpm, the liquid was decanted, and the pellet was resuspended in 35 μl of distilled H_2O. Samples were stored at 4 C.

The polymerase chain reaction (PCR) sample consisted of 2 μl of DNA to 48 μl of master mix containing Takara *Ex* Taq Polymerase (Takara Bio Inc., Seta, Japan). Concentrations were based on the manufacturer's guidelines. Reactions were run on a GeneAmp® PCR System 9700 thermocycler for 50 cycles. Each cycle had 1-min denaturing (94 C), 30-sec annealing (54 C), and 1-min extension (72 C). *Trypanosoma cruzi* genomic DNA (ATCC 30266D, American Type Culture Collection, Manassas, Virginia) served as the positive control.

Amplified DNA was examined on a 1.25% low EEO agarose gel and modified TAE buffer. Samples were run with a 100-bp ladder (Promega, Madison, Wisconsin) at 84 V for approximately 60–100 min, stained using ethidium bromide, and visualized on a UV light table. Bands were cut from the gel, purified using Montage® DNA purifying kit (Millipore, Billerica, Massachusetts), and sequenced at the Rutgers University Biotechnology Center for Agriculture and the Environment.

Resulting sequences were retrieved and edited using the Chromas®Lite software. The chromatograms of sequences were further examined by aligning the forward and reverse sequences. This allowed for filling in any missing base pairs. Sequences were compared with other known sequences in the GenBank database using BLAST® searches. The following conditions were used to determine correct identification. Positive identification to species was made when at least 98% of the base pairs matched to a known sequence, there was only one species that had a 98% match or above, and there were multiple results to the same species. Positive identification to genus was made when there was at least 95% match to a known sequence, and all the top matches belonged to the same genera. If the top matches belonged to various genera within the same class, they were identified to class level only. We did not estimate a positive identification below 92% base pairs.

To determine the phylogenetic relationships of the amplified trypanosome DNA, results were aligned with Tyrpanosomatidae sequences using BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999). The alignment consisted of a 393-bp region of the GAPDH gene. The alignment included 37 sequences from Trypanosomatidae, 9 sequences from field-collected *Cx. territans*, and 2 sequences from the *T. cruzi* control. The outgroup consisted of *Euglena gracilis* and *Bodo saltans*. Our original outgroup for the maximum likelihood analysis was *Lutzomyia longipalpis*. This species was chosen to represent Diptera DNA, because the GAPDH gene was not available for *Cx. territans*. Results showed that our amplified bands were *Trypanosoma* sp. DNA, and not dipteran DNA, so the dipteran outgroup was removed from the analysis.

Maximum likelihood analysis of nucleotide alignments were carried out using PAUP* version 4.0b10 (Swofford, 2005). The model was determined using Modeltest 3.7 software (Posada and Crandall, 1998) and consisted of the general time reversible model (GTR+G) with 4 category gamma distributions. Bayesian analysis was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The general time reversible model was used for analyses of nucleotide sequence alignments. Rate variation across sites was modeled using a gamma distribution. The Markov chain Monte Carlo search was run with 4 chains for 2,000,000 generations, with trees sampled every 100 generations, until the average standard deviation reached 0.007.

Statistical analysis

Data were analyzed using SPSS software version 15.0 (SPSS, Inc., 2005). For the blood meal analysis, a linear regression model was performed to determine whether the rate of blood meal digestion affects the ability to amplify host DNA. Variables included Sella stage of development (WHO, 1975), and percentage of samples resulting in successful host identification. An analysis of variance was performed to determine the affects of location on trypanosome prevalence rates. New Jersey was divided into 3 regions: northern (Bergen and Sussex counties), central (Monmouth, Ocean, and Mercer counties), and southern (Atlantic, Burlington, Camden, Cape May, and Salem counties), and the prevalences were compared. Five-year means of prevalence rates and standard errors were plotted. A curvilinear regression was performed to determine the temporal pattern of trypanosome prevalence rates in *Cx. territans*.

RESULTS

We collected 119 bloodfed *Cx. territans* females from 10 counties in New Jersey, representing the north (Sussex and Bergen counties), central (Monmouth, Mercer, and Ocean counties), and southern (Atlantic, Burlington, Camden, Cape May, and Salem counties) parts of the state. We were able to amplify

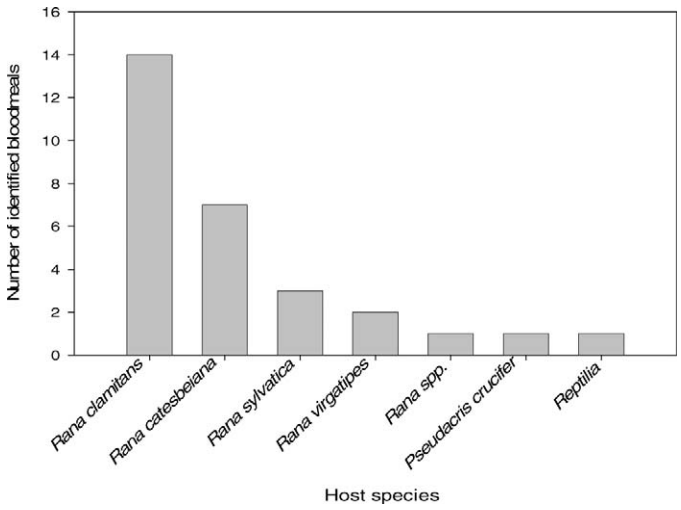

FIGURE 1. Identification of *Culex territans* blood meal hosts, determined by polymerase chain reaction and sequence analysis. Bars indicate the number of individuals that were identified as a vertebrate host species.

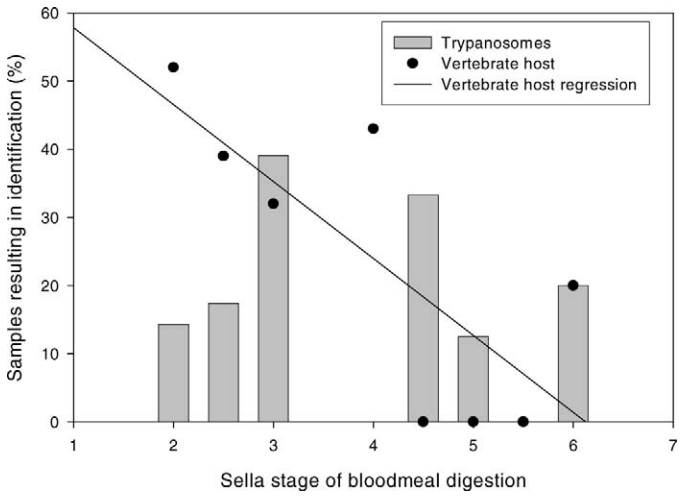

FIGURE 2. Number of successful trypanosome and vertebrate host identifications, from *Culex territans* blood meals, based on sella stage of blood meal digestion, determined by polymerase chain reaction and sequence analysis. Increasing sella stages indicate longer periods of digestion. Bars show the percentage of all samples tested, where we were able to identify trypanosomes in the blood meal. The line shows the percentage of vertebrate hosts we were able to identify within the blood meals at increasing stages of digestion.

the cytochrome b region in 42 of the 119 specimens. Positive vertebrate host identifications were made in 29 of the females. *Culex territans* readily fed on amphibians (Fig. 1), with the highest percentage feeding on *R. clamitans* (48%), followed by *R. catesbeiana* (24%), *R. sylvatica* (10%), *R. virgatipes* (7%), *Pseudacris crucifer* (4%), and *R.* spp. (4%). A single blood meal was identified as an unknown reptile (4%). Amplification of host DNA was most likely when blood meals were in an early stage of digestion (Fig. 2). The ability to amplify host DNA decreased as blood meal digestion increased ($R^2 = 0.61$, $F = 9.17$, $P = 0.02$).

Vertebrate hosts varied throughout the state, with the highest percentage feeding on *R. clamitans* (63%), *R. catesbeiana* (31%), and *Pseudacris crucifer* (6%) in northern New Jersey; *R. clamitans* (100%) in central New Jersey; and *R. sylvatica* (42.8%), *R. virgatipes* (28.6%), *R. catesbeiana* (14.3%), and an unknown reptile (14.3%) in southern New Jersey. Sample size was too small to determine seasonal variation in blood meal hosts. *Rana clamitans* and *R. catesbeiana* were a blood meal source throughout the spring and summer.

Trypanosomes were detected in 24 (20%) of the blood meals examined. In 6 of the samples containing trypanosomes, there was also positive host identification, which included *R. clamitans* (50%), *R. catesbeiana* (16%), *R. virgatipes* (16%), and *Rana* spp. (16%). Amplification of trypanosome DNA was not affected by the stage of blood meal digestion ($R^2 = 0.05$, $F = 0.33$, $P = 0.58$). Trypanosomes were amplified in females that were fully engorged (14.3%) to almost fully gravid (20%).

Samples containing trypanosomes were obtained in each of the 10 counties where *Cx. territans* were examined (Fig. 3). Females with the highest prevalence of trypanosomes occurred in Atlantic (100%) and Monmouth (52%) counties; moderate prevalence occurred in Burlington (33%), Camden (33%), Mercer (25%), and Sussex (25%) counties; and low numbers occurred in Ocean (14%), Salem (10%), Bergen (7%), and Camden (6%) counties. There was no significant difference ($F = 1.01$, $P = 0.417$, df = 2, 9) among prevalences throughout

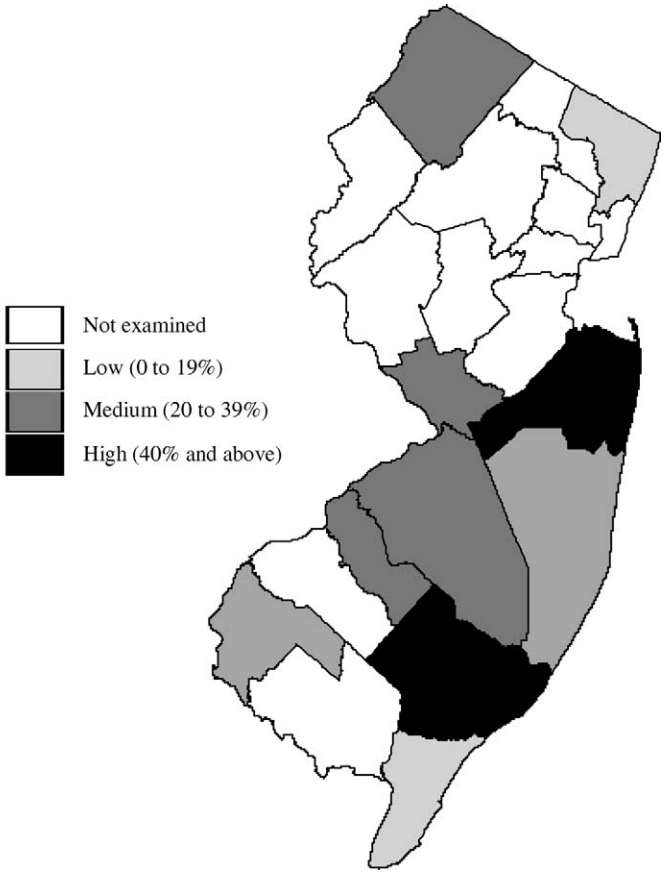

FIGURE 3. Distribution and prevalence of *Culex territans* trypanosomes. Counties that are shaded in white indicate that no samples were submitted. In all counties where samples were submitted, we were able to find trypanosomes in *Cx. territans* blood meals.

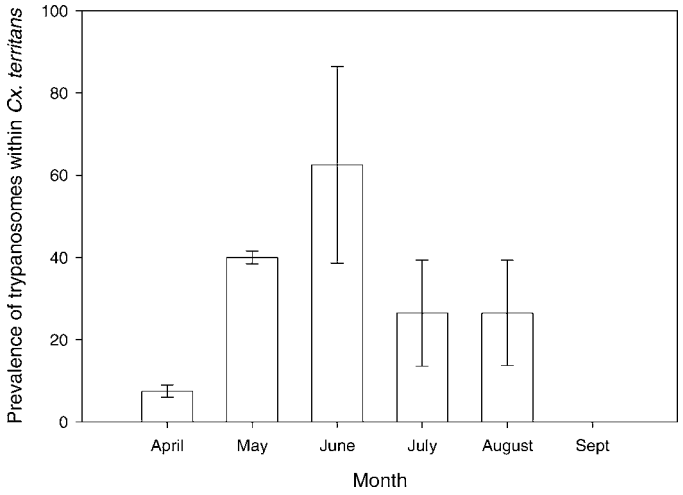

FIGURE 4. Prevalence of trypanosomes (per 100) within *Culex territans* by month. Results represent 5-yr mean percentages and SE for 119 females collected from 2003 to 2007.

northern (13.2 ± 10.4), central (30.3 ± 8.5), and southern (17.1 ± 7.4) New Jersey.

The presence of trypanosomes in the blood meals occurred in a seasonal pattern (Fig. 4). The temporal distribution showed a quadratic trend ($R^2 = 0.897$, $P = 0.033$), where the prevalence of trypanosomes within females increased in the spring, peaked in June, and then decreased toward the end of the season. The first infected *Cx. territans* was collected in April. A higher proportion occurred in females collected in June (37.5%), July (25%), and August (25%). No trypanosomes were collected in September, although 12% of the overall engorged females were collected during this month.

Nine trypanosome sequences were used in the phylogenic analysis, based on the quality of the returned sequence. Inferred phylogenetic trees from the Bayesian and maximum likelihood supported the placement of the aquatic and terrestrial clades into 2 separate groups. Results of our Bayesian analysis (Fig. 5) showed 100% bootstrap support that all of our samples from *Cx. territans* placed in the aquatic clade, and 100% support that 8 of our samples belonged to the group of amphibian trypanosomes, including *T. rotatorium*, *T. fallisi*, and *T. mega*. One of the samples was closely related to a trypanosome isolated from an aquatic leech.

In both the maximum likelihood and Bayesian analyses, the *Trypanosoma* spp. formed a monophyletic group comprising aquatic and terrestrial species. The aquatic clade, which contains amphibian and fish trypanosomes, formed a distinct lineage from the terrestrial *T. brucei* and *T. cruzi* clades. Although samples from *Cx. territans* came from at least 3 species of amphibians, all isolated trypanosomes fell within the aquatic clade. The sample Terr108, grouped with an aquatic leech. We were unable to identify the vertebrate host in this sample, but *Cx. territans* was at an early stage of blood meal digestion. Within the amphibian clade, our trypanosome samples grouped with *T. fallisi* and *T. rotatorium*. The sample Terr066 (*Rana virgatipes* from southern New Jersey) grouped with *T. rotatorium* (100% bootstrap support). Two trypanosomes collected in 2006 from Monmouth County, Terr086 (*Rana* sp.) and Terr087 (*Rana clamitans*), were grouped together (100% bootstrap support). These 2 trypanosomes clustered (92% bootstrap) with remaining samples (Terr107, Terr112, Terr108, Terr117, and Terr113) collected from central New Jersey in 2007. The *T. cruzi* control was sequenced twice. On both occasions, our sequences were correctly placed with *T. cruzi* in our phylogeny (100% bootstrap support).

DISCUSSION

We found *Cx. territans* readily bloodfed on frogs, including *R. clamitans*, *R. catesbeiana*, *R. sylvatica*, *P. crucifer*, and *R. virgatipes*. *Culex territans* has been observed blood feeding from each of these species in nature (Crans, 1970). Blood meals from non-anuran hosts include rabbit and rodent (Crans, 1970), horse, raccoon, American robin, and common grackle (Savage et al., 2007). Although studies by Crans (1970) and Savage et al. (2007) identified amphibian blood meals within *Cx. territans*, this is the first study to identify the species of amphibian from bloodfed females.

The amphibian primer set successfully amplified the cytochrome b gene in all amphibians and reptiles tested. The primer set was designed to amplify all genera within this group, and was deliberately made unspecific to maximize the likelihood of detecting the cytochrome b gene from multiple potential hosts. Because the primers were not specific, multiple errors occurred during sequencing, resulting in 70% positive identification to vertebrate host. Other primer sets were used to determine vertebrate host, but they were less likely to detect amphibian blood meals. The trypanosome primer set was designed to be specific and successfully amplified the *T. cruzi* control in all PCR reactions.

Host preference is affected by temporal and spatial abundance of potential hosts (Savage et al., 1993). Regardless of month and location, the highest proportions of blood meals were from *R. clamitans*. *Culex territans* exists both temporally and spatially with *R. clamitans* (Bartlett-Healy et al., 2009), suggesting that *Cx. territans* acquires blood meals from hosts near their oviposition site. Desser et al. (1973) found that *Cx. territans* were prevalent in their study areas around *R. clamitans*, suggesting that these mosquitoes could be vectors of anuran trypanosomes.

Culex territans contained trypanosomes in 20% of the females examined. Barta and Desser (1984) reported prevalences of *T. ranarum* within *R. catesbeiana* (4%) and *R. clamitans* (10.5%), but prevalences of *T. rotatorium* were high for *R. catesbeiana* (52%) and *R. clamitans* (43.9%). If *Cx. territans* were acquiring *T. rotatorium*, our results should mimic these high prevalences. Instead, our results are similar to those found with *T. ranarum*. This complex is made up of the giant anuran trypanosomes *T. mega*, *T. fallisi*, and *T. ranarum*. These species have been detected in diverse anurans, including *R. catesbeiana*, *R. clamitans*, *R. sylvatica*, and *B. americanus* (Barta and Desser, 1984). The potential vectors for many of the amphibian trypanosomes, including *T. ranarum* and *T. fallisi*, are still listed as unknown. Although *Cx. territans* has never been demonstrated to transmit amphibian trypanosomes, transmission studies using Diptera could elucidate this missing information.

Results showed a seasonal distribution of females infected with Anuran trypanosomes. This seasonal distribution has been documented in the literature for amphibian infections since the

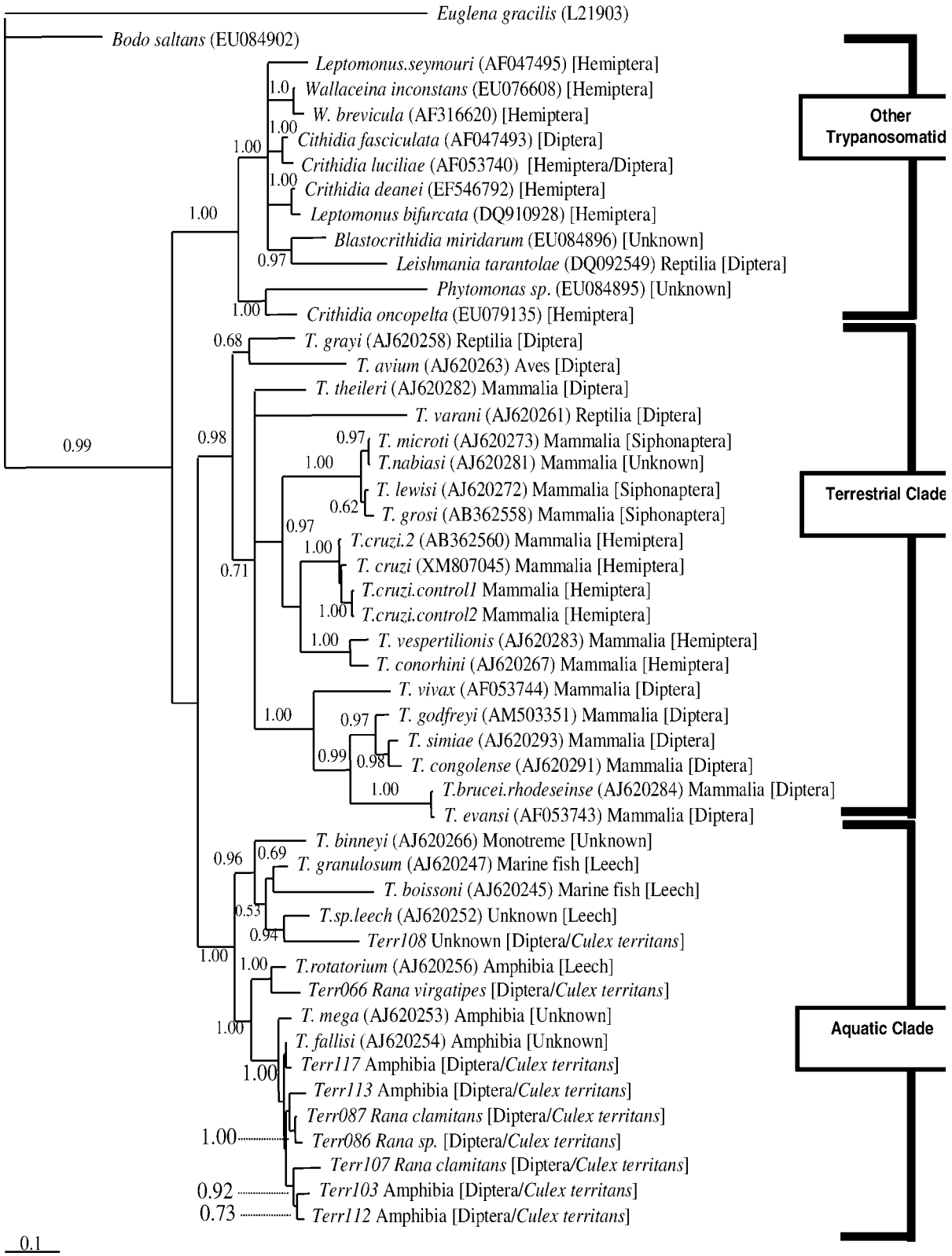

FIGURE 5. Likelihood phylogram of Trypanosomatidae from Bayesian analysis, with placement of *Culex territans* trypanosomes. Bootstrap values of Bayesian analysis are at the nodes. Each branch includes species (accession number) vertebrate host, and [invertebrate host].

1800s (Bardsley and Harmsen, 1973). In most cases, prevalence is highest in spring and slowly decreases throughout the summer. Possible explanations for this distribution have included changes in photoperiod, temperature, amphibian glucose levels, and seasonal distribution of leeches (Bardsley and Harmsen, 1973). Leeches attach to amphibians beginning in May, and they remain attached through June and July (Bardsley and Harmsen, 1973). By August, leeches are less abundant on amphibians, possibly explaining why our infection levels were highest in May, June, and July. Another factor contributing to prevalence in May is that frogs used as blood meals are mature adults that have been exposed to trypanosomes for over a year. As the season progresses, tadpoles are metamorphosing into adults, and by August there is a higher proportion of young adults.

Lack of host specificity increases the probability of being colonized by parasites (Price, 1980). *Culex territans* feeds on a diversity of amphibians, increasing the likelihood of acquiring trypanosomes. Trypanosomes have been detected in all vertebrate classes. For many of these species of trypanosomes, the vector and mode of transmission remains unknown. Recent phylogenies have shown specific clades of trypanosomes, which may be similar by vertebrate host or invertebrate vectors. However, even within a particular clade, the vectors and modes of transmission may differ. Co-speciation does not occur between trypanosomes and their vertebrate and invertebrate hosts, suggesting that invertebrates are capable of transmitting a wide variety of parasites (Hamilton et al., 2007). Instead of co-evolving with invertebrate or vertebrate hosts, trypanosomes likely colonize new hosts based on the traits they possess (Hamilton et al., 2007).

The question arises as to what role *Cx. territans* plays in the trypanosome phylogeny. Leeches are not the only vectors of amphibian trypanosomes. Sand flies transmit trypanosomes to amphibians and reptiles in Brazil (Ferreira et al., 2008), and development to the epimastigote stage in *T. rotatorium* has been shown within *Cx. territans* (Desser et al., 1973), although transmission has never been examined. The trypanosomes isolated from *Cx. territans* occurred within the aquatic clade, splitting from leech-transmitted trypanosomes. The origin of the Culicidae and Phlebotiminae occur at least 40 million yr after the origin of *Placobdella* spp. leeches, suggesting if dipteran transmission of aquatic trypanosomes occurs, then it diverged from leech-transmitted trypanosomes. Our phylogeny supports a potential new lineage of dipteran-transmitted anuran trypanosomes may be diverging from the aquatic clade. Diptera are suitable invertebrate hosts for trypanosomes, with 41% of the lower Trypanosomatidae occurring within this family (McGhee and Cosgrove, 1980).

The goal of the present study was not to implicate *Cx. territans* as a vector of trypanosomes but to determine the blood meal hosts and potential parasites they are acquiring during blood feeding. Although *Cx. territans* has not been demonstrated to transmit trypanosomes to amphibians in the laboratory, they are readily acquiring these parasites during blood feeding. All trypanosomes acquired by *Cx. territans* belonged to a monophyletic group known as the aquatic clade, which includes several vertebrate and invertebrate hosts. A tritrophic study of invertebrate hosts, trypanosome species, and vertebrate hosts can elucidate existing phylogenies.

Our study shows that *Cx. territans* are most likely acquiring *T. ranarum* and *T. fallisi*. Considering the prevalence in which females are picking up parasites, there should be a selective advantage to those trypanosomes that can complete development within the mosquito, suggesting that transmission studies using Diptera should be examined further. Trypanosomes were detected in *Cx. territans* at all stages of blood meal digestion, indicating this species is a suitable invertebrate host for trypanosome development. Trypanosomatidae from *Leptomonas*, *Crithidia*, *Herpetomonas*, and *Trypanosoma* have all been described from mosquitoes (Bates, 1949). Trypanosomes can complete development to the epimastigote stage in *Cx. territans* (Desser et al., 1973), indicating conditions are suitable for development. Development of trypanosomes within an invertebrate host does not necessarily indicate it is a vector but warrants further transmission studies. Our results show that future experiments on amphibian trypanosomes along with vertebrate and invertebrate host studies are necessary and can shed light on the complex phylogenies of Trypanosomatidae.

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