COMPETENCE OF SPATIALLY DISTINCT POPULATIONS OF *AMBLYOMMA AMERICANUM* AS VECTORS FOR *THEILERIA CERVI*

by

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I. INTRODUCTION

Background

It has been speculated that in the last 100 years, tick-transmitted zoonoses in the United States of America have been discovered at a relatively constant rate of one per decade. However, over the last 20 years, the rate of emerging (or re-emerging) tick-borne diseases has increased significantly (Childs et al. 1998). Amid the numerous factors that have been proposed to explain these increases are changes in the genetic relationships among tick vectors, infectious agents, and vertebrate hosts. (Gubler 1998).

McLain et al. (1995) proposed that genetic differences among populations of a tick species might be associated with, or indicative of, the capacity of vector populations to transmit disease. Because of the public and animal health importance of ticks as vectors of infectious agents, evaluation of genetic mechanisms that may affect the ability of acarines to acquire, maintain, or transmit a disease agent is fundamental to understanding arthropod-transmitted diseases (Tabachnick 1992).

Within the United States of America, *Amblyomma americanum* is found primarily in the southcentral and southeastern states (Hair and Bowman 1986) but has been found as far north as New York (Means and White 1997) and Maine (Merten and Durden 2000). In addition to inciting a suppurative, pruritic, and erythematic lesion inflicted by its long hypostome, *A. americanum* transmits several disease agents to domestic and wild animals (Allan 2001) as well as to human beings (Childs & Paddock 2002).

Hilburn and Sattler (1985) employed electrophoretic techniques to examine nine geographically disjunct populations of *A. americanum* for variation at 21 enzyme loci. High identity values among *A. americanum* populations indicated a relatively undifferentiated gene pool. However, advancements in molecular techniques now permit the evaluation of genetic

markers more sensitive than allozyme loci for detecting intraspecific variation among tick populations (Navajas and Fenton 2000).

Eukaryotic ribosomal DNA (rDNA) consists of several hundred tandemly repeated arrays containing genes encoding the 18S, 5.8S and 28S subunits, internal transcribed spacers (ITS) 1 and 2, external transcribed spacer (ETS), and a non-transcribed spacer (NTS) that separates individual repeats (Hillis and Dixon 1991). Due to small deletions and additions that arise via replication slippage, ITS 1 and ITS 2 sequences are highly variable in arthropods (Wesson et al. 1993). Because ITS 1 and ITS 2 regions are less conserved than adjacent ribosomal small subunit genes, they have been used to infer phylogenic relationships among closely related species and subspecies of ticks (Navajas and Fenton 2000).

Vector competency has been defined as the ability of arthropods to acquire, maintain, and transmit microbial agents (Lane 1994). In most instances, the vector competency of ticks has been determined by whether particular species of acarine can transmit an infectious agent. Few studies have quantitatively determined the degree to which a tick species acquires, maintains, and transmits an infectious agent because a means of enumerating infection rates within acarine hosts has not been developed. However, Walker et al. (1979) described a method in which sporozoites of *Theileria parva* and *T. annulata* within hypertrophied salivary glands of *Rhipicephalus appendiculatus* and *Hyalomma anatolicum,* respectively, could be stained and counted.

Theileria cervi is a non-pathogenic intraerythrocytic protozoan parasite that infects white-tailed deer (*Odocoileus virginianus*) in North America with a distribution that overlaps that of its only known vector, *A. americanum* (Kocan and Waldrup 2001). *Theileria cervi* infections in *A. americanum* are initiated when a nymphal tick feeds on an infected white-tailed deer and ingests a blood meal containing piroplasms of the protozoan parasite. After

sexually reproducing within its tick host, *T. cervi* undergoes sporogony within granular E cells of type III acini of *A. americanum* (Hazen-Karr et al. 1988; Kocan et al. 1988). Development of sporozoites in *A. americanum* results in hypertrophied acini that are easily distinguished from uninfected cells. Enumeration of *T. cervi* within the salivary glands of *A. americanum* provides both a qualitative and quantitative assessment of infection in which the prevalence, abundance, and intensity of the parasite within its tick hosts can be compared (Laird et al. 1988).

Because tick-transmitted infectious agents of wildlife are often well established in their natural hosts and the parasites commonly cause clinical disease only in stressed individuals (Kocan and Kocan 1991), investigations that quantify vector/infectious agent relationships in wildlife species may serve as models for tick transmitted diseases in general. Phylogenetic analysis of genetic differences between populations of *A. americanum*, coupled with experimental infections with *T. cervi* offers a unique opportunity to evaluate the association of genetic variation between arthropod populations and their ability to vector an infectious agent. Data from the present study should prove helpful in evaluating spatial and/or temporal changes that impact disease prevalence as well as provide a means of determining risk factors related to disease occurrence.

Objectives

I. Phylogenetically compare levels of genetic variation between two spatially distinct populations of *Amblyomma americanum* as judged by differences in their ITS 2 nucleotide sequences.

II. Qualitatively and quantitatively determine the capacity of two genetically characterized populations of *Amblyomma americanum* to acquire, maintain, and transmit *Theileria cervi* to white-tailed deer.

II. MATERIALS AND METHODS

Experimental Design

The present study was conducted in two phases. First, molecular techniques and phylogenetic analyses were used to sequence and compare the rDNA ITS 2 region of spatially distinct populations of *A. americanum*. Second, representatives from the genetically characterized populations of *A. americanum* were allowed to feed on *T. cervi*-infected white-tailed deer and measurements of their engorgement weight and molting success were compared; in addition, their ability to acquire, maintain, and transmit the protozoan parasite were determined.

Sources of *Amblyomma americanum* eggs

Non-fed laboratory-reared adult *A. americanum* (Colony) were obtained from the Tick Rearing Laboratory at Oklahoma State University (Stillwater, OK). The colony was established from wild *A. americanum* collected originally in Oklahoma and has been maintained for over 30 years. As such, this colony of *A. americanum* was considered spatially distinct from wild tick populations. Infusion of new *A. americanum* into the Tick Rearing Laboratory is made at irregular intervals, less than once a year.

Non-fed *A. americanum* adults (Wild) were collected from Western Hills State Park in Cherokee County (36^0 00' N, 96^0 00'W) in eastern Oklahoma by use of carbon dioxide traps (Patrick and Hair 1978). According to records of the Entomology Museum at Oklahoma State University (OSU), which was founded in 1954, *A. americanum* was well established in eastern Oklahoma at that time. Because the movement of animal hosts and ticks into and out of Western Hills State Park is not restricted, the gene flow of *A. americanum* collected there was considered representative of a wild population.

Twenty pairs of non-fed adult *A. americanum* from each tick population (Colony and Wild) were placed in separate stockinette cells (3M Health Care, St. Paul, MN) attached to

shorn domestic sheep, and allowed to feed to repletion. Replete female *A. americanum* were transferred from their stockinette cells into paper cartons and maintained separately. Both groups of ticks were stored in a humidity chamber (90 to 98% relative humidity at 25 C with a 14-10 hr light-dark photophase) and allowed to oviposit.

Extraction, Amplification, and Sequencing of *Amblyomma americanum* DNA

Following oviposition, female *A. americanum* were removed from the cartons and egg masses were uniformly mixed by stirring with a tongue depressor. DNA was extracted from 10 mg of late developmental-stage eggs from each population of *A. americanum* (McLain et al. 1995). The DNeasy tissue kit (Qiagen, Valencia, CA) was used for extraction of *A. americanum* DNA. Based on the advice of the manufacturer, the protocol for insects was used for DNA isolation since no procedure was available for use with ticks. Multiple DNA extractions were performed on eggs derived from both tick populations.

Oligonucleotide primers 7923 and 7924 designed by Zahler et al. (1995) to target the conserved and flanking 5.8S and 28S rRNA regions (Table 1; Figure 1) along with PCR were used for amplification and sequencing of the ITS 2 region from tick genomic DNA. Amplifications were performed in 50 µl volumes containing 5 µl 10X *Taq* buffer (Promega, Madison, WI), 2.5 µl genomic DNA, 2.5 µl dimethylsulfoxide, 2 mM $MgCl_2$, 10 mM each dNTP (Promega), 25 pmol each primer, and 2.5 U *Taq* polymerase (Promega). Cycling parameters were an initial denaturation at 96 C for 3 min followed by 30 cycles of denaturation at 94 C for 1 min then annealing at 51 C for 1 min and extension at 72 C for 2 min. Following the 30 cycles, there was a final extension at 72 C for 10 min. Amplified products (5 µl) were separated on 1.5% agarose gels, stained with ethidium bromide, and examined by ultraviolet illumination.

Table 1. Oligonucleotide primers used to amplify and sequence the rDNA ITS 2 region of *Amblyomma americanum*

Primer number	Primer sequence (5' —> 3")	Reference
7923	CGGGATCCTTC(A/G)CTCGCCG(C/T)TACT	Zahler et al. 1995
7923	CCATCGATGTGAA(C/T)TGCAGGAC	Zahler et al. 1995
8483	TACGTGTAGCCGAACT	New
8484	GGATAACGCAGAGAGT	New
8659	GCCGTCGGTCTAAGTGCTTCGCAGTCTC	New
8660	GCCCTGCGCAGACGACGTAAC	New
8722	CAGCAGTTCGGCTACACGTACGCGGG	New
8769	CTTCGGAGGCCAGACGCTCTACG	New
8770	GGCTCGAAACTCTCTGCGTTATC	New

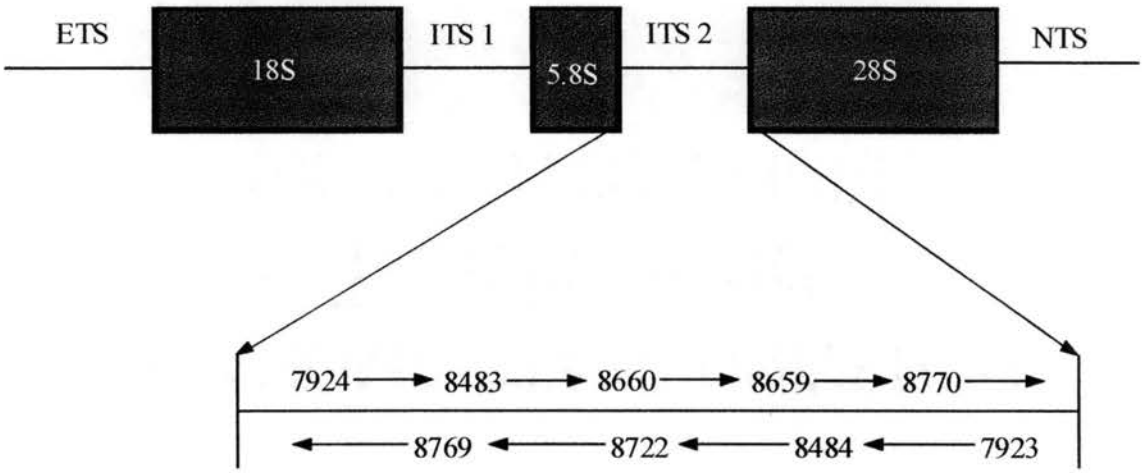

Figure 1. Graphic representation of the rDNA gene array and positions of oligonucleotide primers used to amplify and sequence the ITS 2 region of *Amblyomma americanum*.

PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced at the Oklahoma State University Recombinant/DNA Protein Research Facility (Stillwater, OK) using a Model 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). In total, nine primers (Table 1; Figure 1) were used to obtain complete overlapping forward and confirmatory reverse strands of the ITS 2 region. Multiple PCR products of individual DNA extractions from eggs of mixed-parentage from both Colony and Wild *A. americanum* populations were purified and sequenced. To prevent contamination of samples, PCR master mix assembly, amplification, and DNA purification were performed in separate areas. Positive displacement pipettes and aerosol-free pipette tips were also used to further guard against contamination.

Phylogenetic Analysis

Contiguous, overlapping fragments within individuals were assembled using AssemblyLIGN ™ (Oxford Molecular Group PLC 1998). Multiple sequences were aligned using CLUSTALX (Thompson et al. 1997) and subsequently imported into MacClade (Madison and Madison 2000) for visual inspection. Nucleotides were coded as unordered, discrete characters (G, A, T, C) and gaps as missing data. Prior to phylogenetic analysis the most appropriate model of DNA evolution was determined using Modeltest (Posada and Crandall 1998). Tamura and Nei-corrected genetic distances (as determined by Modeltest) were calculated among all sequences. A neighbor-joining tree (Saitou and Nei 1987) rooted with an ITS 2 sequence of *A. vikirri* (GenBank accession number AF199112) was constructed based on the corrected distance values, the minimum evolution criterion, and 250 bootstrap iterations.

Sources of White-tailed Deer

Five white-tailed deer were obtained from the Oklahoma Department of Wildlife Conservation or the Animal Damage Control, Oklahoma Department of Agriculture, as young-of-the-year orphans. Deer were housed at the OSU Wild Animal Research Facility at Camp Redlands (Stillwater, OK) or in tick-free isolation facilities at the OSU Tick Rearing Laboratory. Facilities and procedures used in this study were approved by the OSU Institutional Animal Care and Use Committee.

***Amblyomma americanum* Infestations on White-tailed Deer**

Experimental and control infestation of deer with *A. americanum* were conducted as shown in Figure 2. Nymphal *A. americanum* from each population were sequentially fed on two *T. cervi*-infected deer (Donors 1 and 2) on separate occasions. Donor 1 had an average *T. cervi* parasitemia of 3.5% and 5.1% when Colony and Wild ticks were acquisition fed, respectively. Donor 2 had an average *T. cervi* parasitemia of 3.6% and 11.0% at acquisition feedings of Colony and Wild ticks, respectively. To ensure ticks from a previous infestation did not contaminate subsequent feedings, deer were housed in isolation for a minimum of two weeks between trials. Two *T. cervi*-naive deer (Principals A and B) were used for transmission feeding and one *T. cervi*-naive deer served as an uninfected control.

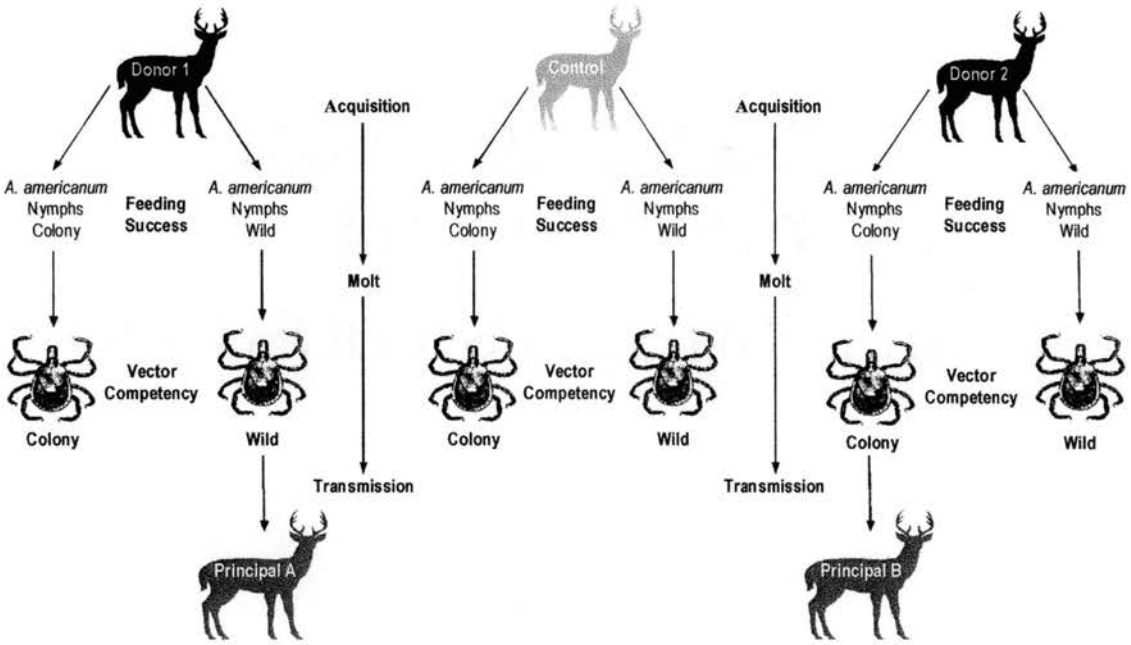

Figure 2. Experimental design of *Amblyomma americanum* acquisition feedings on *Theileria cervi* infected deer (Donors 1 and 2) and transmission trials to naive deer (Principals A and B). Timing and frequency of measurements assessing tick feeding success and vector competency are noted.

Procedures for *A. americanum* experimental and control infestations followed those of Ewing et al. (1995). Briefly, deer were tranquilized via intramuscular injection with xylazine (2 mg/kg body weight; Vedco, St. Joseph, MO) and placed in a rubber-coated expanded metal cage (2.0 x 1.7 x 1.1 m) housed in isolation. Approximately 500 *A. americanum* nymphs from a population were placed between the shaved shoulder blades of the deer. To confine and minimize escape of ticks during the attachment phase, the torso of deer were fitted with a stockinette that was secured in place with masking tape at the time of infestation. The stockinette was removed after 12 hours. Cages were placed over metal pans containing tap water that acted as a moat and trapped ticks as they reached repletion and fell off their host. Margins of the pans were lined with double-sided tape to prevent the escape of ticks from the water moats. Replete ticks were collected daily. Food and water were provided *ad libitum*.

Determination of *Theileria cervi* Infection in Deer

Blood samples were collected from deer at various times throughout the study. Sample collection followed procedures described by Ewing et al. (1995). *Theileria cervi* infection in deer was confirmed by microscopic observation of piroplasms in erythrocytes on thin blood smears stained with Diff-Quik stain (American Scientific, McGaw Park, IL). *Theileria cervi*-naive deer were identified by the absence of piroplasms in deer blood after repeated examination. To determine patent infection of *T. cervi* in Principals A and B during transmission trials, thin blood smears were examined every other day post-infestation until piroplasms were detected.

Determination of Feeding and Molting Success

Feeding success of Colony and Wild populations of *A. americanum* was assessed at the end of each acquisition infestation by measuring engorgement weight of replete nymphs.

Replete nymphs were weighed to the nearest 0.1 mg using a Model AB204 electronic balance (Mettler Toledo, Columbus, OH). Percentage of replete nymphs that molted to adults was also determined.

Vector Competency

Assessment of potential vector competency was based on enumeration of *T. cervi*-infected salivary gland acini (Figure 3) in Colony and Wild populations of *A. americanum* and followed procedures described by Laird et al. (1988). To stimulate development of *T. cervi* in salivary glands, approximately 40 pairs of molted-adult ticks from a population were placed in a stockinette cell attached to shorn domestic sheep and allowed to feed for 6 days prior to dissection. Dorsal and ventral halves of the exoskeleton of fed ticks were separated with a razor blade under magnification. Salivary glands were dissected on to a microscope slides with fine forceps, air dried, stained with methyl green-pyronin, and examined for the presence of hypertrophied acini by light microscopy at 40 x magnification (Walker et al., 1979). The prevalence, abundance, and intensity of *T. cervi* infection as defined by Bush et al. (1997), was determined for both tick populations.

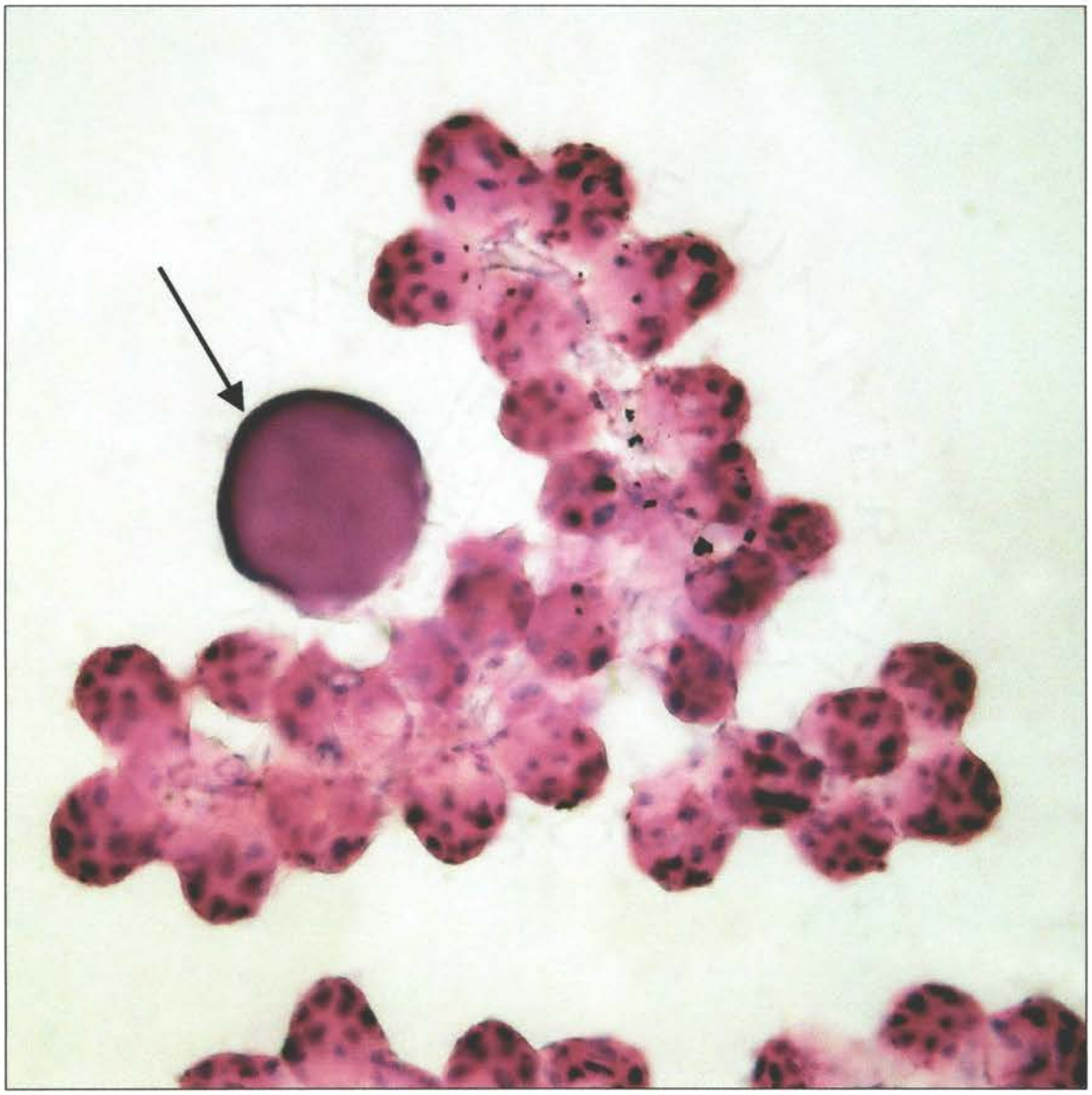

Figure 3. Photomicrograph of methyl green-pyronin-stained salivary glands of *Amblyomma americanum*. The hypertrophied *Theileria cervi*-infected acinus (arrow) is easily identified due to its large size that resulted from sporogonic development of the protozoan parasite.

Statistical Analyses

The proportion of recovered nymphs that molted into adults and the prevalence of *T. cervi*-infected salivary gland acini between Colony and Wild *A. americanum* were compared using a Chi-square test of independence (Sokal and Rohlf 1997). Data for engorgement weight of replete nymphs and the abundance and intensity of *T. cervi*-infected adult *A. americanum* acini were compared by Analysis of Variance following the Kolmogorov and Smirnov test for normality (Sokal and Rolff 1997). Experiment-wise error was controlled using Tukey's method for multiple comparisons (Sokal and Rolff 1997). Data for abundance and intensity of *T. cervi*-infected adult *A. americanum* acini were log10 transformed.

Spearman's Rank correlation was performed to determine if there was an association between the average *T. cervi* parasitemia during acquisition feeding and feeding success and vector competency of Colony and Wild populations of *A. americanum*. The Bonferroni adjustment for multiple comparisons was used as the criterion of statistical significance for Chi-square test of independence and Spearman's Rank correlation (Sokal and Rolff 1997).

III. RESULTS

Amblyomma americanum Genetics

The entire ITS 2 region of *A. americanum* from four and six independent DNA extractions and PCR amplifications representing mixed-parentage samples from the Colony and Wild tick populations, respectively, was sequenced. All samples of *A. americanum* except Wild B 1 resulted in ITS 2 sequences of 1,144 nucleotides in length. The ITS 2 sequence from Wild B 1 was 1,145 nucleotides in length. Internal Transcribed Spacer 2 DNA sequences from all 10 samples were deposited in GenBank (accession numbers AF548530 – AF548539). Of the 1,145 aligned positions, 12 (1%) were variable (Table 2). Aligned positions 147 and 545 reveal fixed transversion substitution differences between the Colony and Wild populations of *A. americanum.* Of the remaining 10 variable sites, only the variation at position 527 provides additional support for differentiation between the two populations of *A. americanum.* At position 527, the Colony population was fixed for G whereas the five samples from the Wild population were polymorphic, with both G and A occurring. Of the remaining nine-nucleotide sites, four (positions 480, 483, 516, 565) reflect additional variation shared between two or more mixed-parentage samples, whereas the remaining five (positions 263, 436, 523, 525, 588) showed variation in only one of the samples (Table 2).

Phylogenetic relationships among the 10 ITS 2 sequences of *A. americanum* are shown in Figure 4. Although minimal levels of nucleotide substitution were detected in ITS 2 sequences of *A. americanum*, bootstrap analysis provided strong support (71% and 80%) for the conclusion that DNA sequences from *A. americanum* populations clustered into separate and distinquishable groups.

Table 2. Intraspecific polymorphisms of rDNA ITS 2 sequences from Colony and Wild populations of *Amblyomma americanum*.

Sequence Name [a]	Base Position											
	147	263	436	480	483	516	523	525	527	545	565	588
Colony A 1	C	-	G	G	G	G	G	G	G	T	D	G
Colony A 2	·	-	A	A	A	R	·	·	·	·	G	R
Colony B 1	·	-	·	·	·	·	·	·	·	·	R	R
Colony B 2	·	-	·	S	S	R	·	·	·	·	R	R
Wild A 1	G	-	·	·	·	R	C	C	R	G	R	R
Wild B 1	G	A	·	·	·	·	·	·	R	G	R	R
Wild B 2	G	-	·	·	·	R	·	·	R	G	R	R
Wild B 3	G	-	·	·	·	R	·	·	R	G	R	R
Wild C 1	G	-	·	·	·	R	·	·	R	G	R	R
Wild C 2	G	-	·	·	·	R	·	·	R	G	R	R

Insertion/deletion events are indicated with a dash (-), whereas dots (·) indicate that the nucleotide is identical to that found in the sequence of Colony A 1. Nucleotides are coded according to the standard International Union of Biochemistry code.

[a] Sequence names begins with population of origin (Colony or Wild) followed by a letter (A, B, or C) that denotes separate DNA extractions and ends with a number (1, 2, or 3) representing different PCR amplifications and sequences.

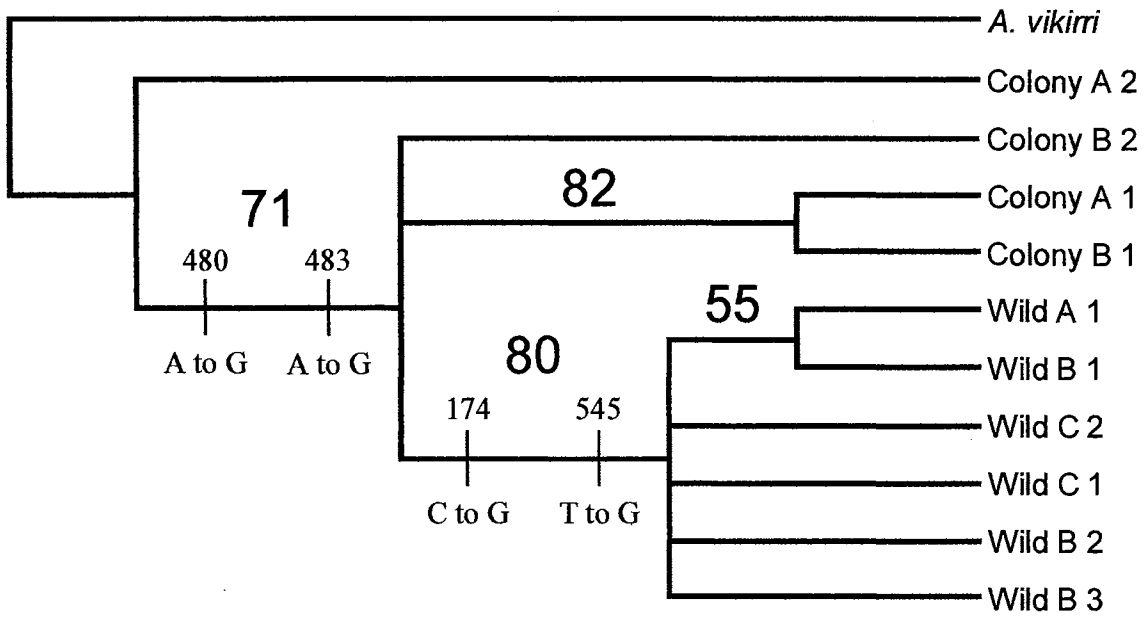

Figure 4. Topology of neighbor-joining bootstrap tree based on Tamura and Nei-corrected ITS 2 sequence differences from Colony and Wild *Amblyomma americanum*. Numbers in large font above internal lineages are the percentages of 250 bootstrap iterations in which each clade was detected. Numbers in smaller-sized font along internal lineages indicate character-state changes of the aligned sequences that unambiguously unite various clades. Branches are not drawn to reflect the proportional change among lineages.

Feeding and Molting Success

Five hundred nymphs from both Colony and Wild populations of *A. americanum* were fed on each Donor and the Control deer. Of these, 33.3% (500 of 1500) of the Wild and 26.1% (391 of 1500) of the Colony ticks were recovered. Comparison of engorgement weights (± standard error) showed that Wild *A. americanum* nymphs (9.04 ± 0.16) were significantly heavier ($F = 14.42$; $df = 2, 885$; $P = <0.001$) than Colony ticks (7.40 ± 0.16). Multiple comparisons of nymphal engorgement weights within Colony and Wild populations of *A. americanum* revealed significant differences depending upon the deer on which they fed (Table 3). Within Colony *A. americanum*, nymphs that fed on Donor 1 (9.70 ± 0.34) were significantly heavier than ticks recovered from Donor 2 (6.962 ± 0.28) or the Control (7.09 ± 0.22). Wild *A. americanum* nymphs that fed on the Control deer (10.34 ± 0.22) were significantly heavier than ticks recovered from either Donor 1 (8. 07 ± 0.21) or Donor 2 (7.54 ± 0.50). A negative correlation was detected between the average *T. cervi* parasitemia during acquisition feeding and the engorgement weight of Wild *A. americanum* ($r_s = -0.359$; $t = 8.895$; $df = 2, 889$; $P = <0.001$).

No difference was detected in the percentage of recovered acquisition-fed Colony or Wild *A. americanum* nymphs recovered that molted to adults. Ninety-two percent (360 of 391) of Colony *A. americanum* acquisition-fed nymphs successfully molted whereas 91% (456 of 500) of Wild ticks molted.

Table 3. Results of Tukey's pairwise comparisons of engorgement weights of Colony and Wild *Amblyomma americanum* nymphs recovered from infested white-tailed deer.

***Amblyomma americanum* Population**	**Comparison**	**P <0.05**
Colony	Donor 1 vs Control	Yes*
	Donor 1 vs Donor 2	Yes*
	Donor 2 vs Control	No
Wild	Donor 1 vs Control	Yes*
	Donor 1 vs Donor 2	Yes*
	Donor 2 vs Control	No

* Statistically Significant

Vector Competency

The prevalence, mean abundance, and mean intensity of *T. cervi*-infected *A. americanum* for both Colony and Wild populations are shown in Table 4. The prevalence of *T. cervi* infection in Colony *A. americanum* was 88.2% and the mean abundance (standard error) was 22.2 (4.8); whereas, the prevalence of *T. cervi* infection in Wild *A. americanum* was 82.9% and the mean abundance was 23.3 (5.4). No difference was detected in the prevalence or mean abundance of *T. cervi* infections with *A. americanum* between tick populations, sex, or Donor. Mean intensity of *T. cervi* infection did not vary between Colony (25.2 ± 5.3) and Wild (27.1 ± 6.1) populations of *A. americanum* (Table 5). However, mean intensity of *T. cervi* infections in female *A. americanum* (36.9 ± 6.6) was significantly higher ($P = 0.007$; df = 1, 101; $P = 0.007$) than in male ticks (11.44 ± 3.0).

In the case of Wild *A. americanum,* mean intensity of infection with *T. cervi* was found to vary depending upon which deer the ticks were acquisition fed on. Wild *A. americanum* acquisition fed on Donor 2 (32.4 ± 11.0) were more heavily infected ($P = <0.05$) than cohorts that fed on Donor 1 (25.2 ± 7.9). No difference was detected in the mean intensity of *T. cervi* infection in Colony ticks irrespective of which Donor they acquisition fed on.

Table 4. Prevalence, mean abundance, and mean intensity of *Theileria cervi* infections in adult Colony and Wild *Amblyomma americanum* acquisition fed as nymphs.

Tick Population	White-tailed Deer	Sex	Sample Size	Prevalence	Mean Abundance	Mean Intensity
Colony	Donor 1	Male	2	50.0	0.5 (0.5)[A]	1.0 (0.0)
		Female	9	88.8	18.8 (11.3)	21.3 (12.6)
	Donor 2	Male	21	85.7	9.1 (3.1)	10.7 (3.5)
		Female	19	94.7	40.6 (10.2)	42.8 (10.5)
	Control	Male	10	0	0	0
		Female	10	0	0	0
Wild	Donor 1	Male	19	84.2	9.9 (5.2)	11.6 (6.0)
		Female	25	88.0	31.4 (11.6)	35.7 (12.9)
	Donor 2	Male	8	87.5	12.8 (6.8)	14.6 (7.6)
		Female	24	79.2	29.5 (11.5)	37.3 (37.3)
	Control	Male	10	0	0	0
		Female	10	0	0	0

[A] Standard Error

Table 5. ANOVA results comparing the mean intensity of *Theileria cervi* infections in experimentally infected *Amblyomma americanum*. Data were log10 transformed and tested for normality using the Kolmogorov and Smirnov test.

Treatment	df	Sum of Squares	Mean Square	F	P
A. americanum Population	1	0.7646	0.7646	2.22	0.140
Donor	1	1.3042	1.3042	3.78	0.055
Sex	1	2.6240	2.6240	7.61	0.007*
Population x Donor	1	1.4441	1.4441	4.19	0.043*
Population x Sex	1	0.2327	0.2327	0.67	0.413
Donor x Sex	1	0.0075	0.0075	0.02	0.883
Population x Donor x Sex	1	0.0107	0.0107	0.03	0.861
Error	101	34.8339	.3449		
Total	108				

* Statistically significant

Further analysis of the mean intensity of *T. cervi* infected female *A. americanum* showed significant differences in the proportions of heavily infected ticks between Colony and Wild populations. The majority of *T. cervi* infected female *A. americanum* from both Colony and Wild populations, 61% and 67% respectively, had 1 to 30 infected acini per infected tick. A significantly higher ($X^2 = 4.196$; df = 1; P = 0.04) proportion of female Colony *A. americanum* 39% were infected with 31 to 170 infected acini per infected tick compared to 23% of Wild ticks of the same classification. Furthermore, the proportion of female Wild *A. americanum* with >170 *T. cervi* infected acini per infected tick (10%) was significantly higher ($X^2 = 8.526$; df = 1; P = 0.004) than that of Colony ticks of the same classification (0.0%; Figure 5). No such difference was seen in male Colony and Wild ticks. No correlation was detected in the average *T. cervi* parasitemia of Donors 1 or 2 and any variable used to estimate vector competency of Colony and Wild populations of *A. americanum*.

Theileria cervi Transmission by *Amblyomma americanum*

Colony and Wild *A. americanum* were both capable of transmitting *T. cervi* to naive white-tailed deer. Principal A had a 3.5% *T. cervi* parasitemia 15 days post-infestation with Colony *A. americanum*, acquisition fed as nymphs on Donor 1. Principal B had a 4.1% *T. cervi* parasitemia 13 days post-infestation with Wild *A. americanum*, acquisition fed as nymphs on Donor 2.

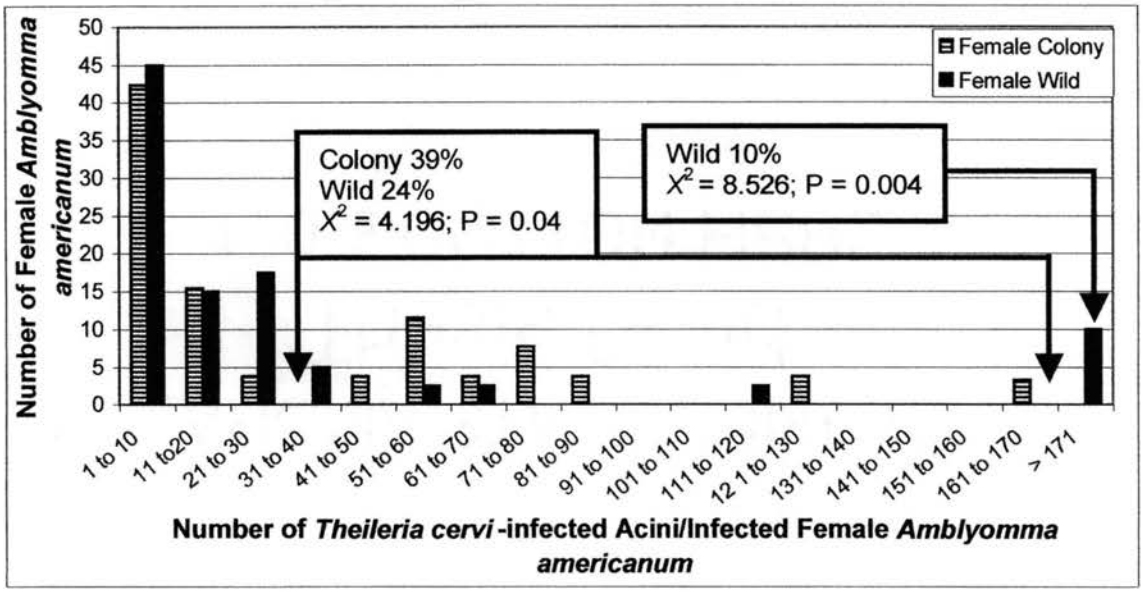

Figure 5. Intensity of Colony and Wild female *Amblyomma americanum* infected with *Theileria cervi*.

IV. DISCUSSION

The ability of ticks to acquire, maintain, and transmit infectious agents can, in part, be influenced by genetically determined intrinsic factors that govern parasite infection in a vector (Lane 1994). As evolutionary forces operate on ticks and the infectious agents they transmit, the genetic make-up of both organisms may be altered. Genetic variation between populations of a tick species could be associated with differences in their capacity to vector infectious agents.

In the present study, comparison of ITS 2 nucleotide sequences from two spatially distinct populations of *A. americanum* revealed distinct genetic variation, with strong support for intraspecific identification of each population. The ITS 2 sequence variation observed between Colony and Wild *A. americanum* was comparable to that reported from other tick species. McLain et al. (1995) identified 35 sites of sequence variation in the ITS 1 region among 20 geographically distinct populations of *Ixodes scapularis* in the eastern United States. Based upon observed patterns of variation, McLain et al. (1995) suggested that some populations of *I. scapularis* were evolving independently owing to restricted gene flow between geographic regions in the tick's range. Wesson et al. (1993) had earlier reported that ITS 1 and 2 nucleotide sequences were variable in spatially distinct populations of *I. scapularis*. By comparing ITS 2 sequences, Rich et al. (1997) showed that *I. ricinus*-like ticks exhibited a considerable degree of intraspecific variation (~4-5%). Conversely, Poucher et al. (1999) and Fukunaga et al. (2000) reported small to negligible differences in the ITS 2 region between individual *Ixodes* spp. Fukunaga et al. (2000) sequenced and analyzed the entire ITS 2 region whereas, Poucher et al. (1999) estimated genetic variation in *Ixodes* spp. through restriction enzyme analysis, which is insensitive to point mutations except at recognition sites of ITS 2 amplicons. Comparison of ITS 2 sequences of *R. sanguineus* by Zahler et al. (1997) revealed little to no intraspecific variation. Similarly, Zahler et al. (1995) sequenced the ITS 2

region of *Dermacentor reticulatus* and reported four sites of intraspecific variation that corresponded to a rate of 0.4%, with none of the variable sites specific for either sex or geographic origin.

Rich et al. (1997) sequenced and compared multiple cloned ITS 2 copies from two individual *Ixodes ricinus*-like ticks to those from disparate populations in eastern North America. The sequence variation from each of the two individual *Ixodes* ticks was nearly half as great as that reported from the geographically disparate populations a finding that did not permit assessment of phylogenetic relationships among the ticks. They cautioned that reasonable steps must be taken to ensure that inferred relationships among ticks are derived from appropriate, orthologous comparisons. In the present study, DNA was extracted from late developmental-stage eggs of mixed parentage to obtain as much genetic information as possible in one sample. This approach was selected instead of extracting DNA from individual ticks because it was more cost-effective and DNA from hundreds of individual tick embryos could be processed at one time. This approach allowed the identification of polymorphic sites between the two *A. americanum* populations that might have been missed had DNA been sequenced from individual larval, nymphal, or adult ticks from each population. By extracting DNA from a pooled sample of embryonated tick eggs, some pitfalls were avoided; however, the use of this approach made it impossible to calculate allelic frequencies in the populations studied.

Taq polymerase has an error rate of 2×10^{-5} errors per base pair (Promega). Therefore, false indications of intraspecific variation, especially point mutations, can result from *Taq* polymerase error (Zahler et al. 1995). Thus, in the present study, an error of 0.2 of the 11,441 base pairs sequenced may be due to misincorporation by *Taq* polymerase. Even if such and error occurred, there was still greater intraspecific variation observed than that

which can be explained by *Taq* error. Furthermore, misincorporation of nucleotides by *Taq* polymerase would be expected to occur randomly throughout the sequenced region. Variable sites in the present study were clustered within a region where other variation was detected. No variation was detected in the sequences from position 1 – 147 or 589 – 1, 145.

The molecular data in the present study is the first report of intraspecific variation between populations of *A. americanum*. Identification of genetic variation between two distinct populations of *A. americanum* should provide a valuable first step in evaluating spatial and/or temporal changes in populations of this tick.

Comparison of engorgement weights from acquisition fed nymphs showed that Wild *A. americanum* were significantly heavier than Colony ticks and that in four of six trials, ticks fed on parasitemic deer were significantly heavier than those fed on the unifected Control. Results of the present study are comparable to those of Randolph (1991) which studied the feeding and survival of *I. trianguliceps* infected with *Babesia microti*, a piroplasm related to *T. cervi*, and reported that ingestion of *B. microti* increased the engorgement weights of *I. trianguliceps*. Reduced engorgement weights negatively impacts the biotic potential of *A. americanum* by suppressing the number of ticks available for future infestation (Garris et al. 1979). In the present study, the significant increase in engorgement weight did not carry over to an increase in the number of Wild nymphs that molted to adults as compared to those of Colony ticks.

In the present study, a significant difference in the mean intensity of *T. cervi*-infected acini in male and female *A. americanum* was observed. A significantly higher ($X^2 = 4.196$; df = 1; P = 0.04) proportion of female Colony *A. americanum* 39% were infected with 31 to 170 infected acini per infected tick compared to 23% of Wild ticks of the same classification. Furthermore, the proportion of female Wild *A. americanum* with >170 *T. cervi*-infected acini

per infected tick (10%) was significantly higher ($X^2 = 8.526$; df = 1; P = 0.004) than that of Colony ticks of the same classification (0.0%; Figure 5). Laird et al. (1988) examined the prevalence, abundance, and intensity of *T. cervi*-infected *A. americanum* in wild-collected and experimentally infected ticks. Sixteen percent (14 of 87) of wild-collected female *A. americanum* were infected with *T. cervi* whereas no male ticks collected from the wild were infected. Laird et al. (1988) also reported that female *A. americanum* under experimental conditions were more heavily infected with *T. cervi* than males. Buscher and Tangus (1986) found, similarly, that experimentally infected female R. *appendiculatus* became more heavily infected with *T. parva* than males. The results of the present study are comparable to those of Laird et al. (1988) and Buscher and Tangus (1986) and indicate that female ticks fed on parasitemic hosts become more heavily infected with *Theileria* spp. than do male ticks.

In the present study, no correlation was observed between parasitemia of *T. cervi.* infected deer and the prevalence, intensity, or abundance of the protozoan parasites in ticks. Laird (1988) suggested that a minimum parasitemia of 1.0% in white-tailed deer was necessary for *A. americanum* to become infected with *T. cervi.*

Purnell et al. (1974) evaluated various conditions that contributed to high infection rates of R. *appendiculatus* with *T. parva.* Although they found considerable variation in the degree of infection of *T. parva* in R. *appendiculatus*, Purnell et al. (1974) were unable to draw any conclusions about casual factors. They suggested that the variation observed in the infection rate of *T. parva* in R. *appendiculatus* could be explained by the juxtaposition of infected gut-epithelial cells and developing salivary glands during the nymphal molt rather than to parasitemia of host on which ticks engorged.

Buscher and Tangus (1986) reported that there are genetic factors that may influence the susceptibility of R. *appendiculatus* to infection with *T. parva.* Although not constant for

every strain of ticks studied, Buscher and Tangus (1986) identified strains of R. *appendiculatus* that consistently became more heavily infected. The present study demonstrated distinct genetic variation between two spatially distinct populations of *A. americanum* and revealed differences in several parameters used to evaluate the feeding success and competence of Colony and Wild ticks to vector *T. cervi*. Wild *A. americanum* infected with *T. cervi* exhibited higher engorgement weights as nymphs and were the only ticks to have >170 infected acini per infected tick. Significant increases in engorgement weight and intensity of *T. cervi* infections in Wild *A. americanum* could increase the biotic potential of the protozoan parasite and enhance the capacity of this tick population to vector the protozoan parasite.

The findings in the present study support the hypothesis that genetically distinct populations of the same species of tick can vary in their ability to vector an infectious agent. The influence that this variation has on the occurrence, distribution, and risk factors related to newly recognized or emerging tick-borne disease of animals and humans has yet to be determined.

The present study identified genetically distinct populations of *A. americanum* and differences in the competence of these two populations to vector *T. cervi*. To further validate these findings, other spatially distinct tick populations should be evaluated throughout North America. Additionally, incorporation of other genetic markers, mitochondrial DNA and/or microsatellites, complementing ITS 2 sequences could enhance the ability to reveal intraspecific variation among ticks and would allow for additional support in determining phylogenies. However, at present, genetic markers (i. e., microsatellites) that have been shown to be highly variable in some taxa, have not been shown to be equally so in all arthropods (Fagerberg et al. 2001).

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VITA 2

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