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Jasmine Hensley April 14, 2015

Prevalence of piroplasmids in ticks from active bobcat territories

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Environmental Sciences

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Abstract

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The tick-borne piroplasmids *Babesia, Theileria,* and *Cytauxzoon* spp. are agents of both human and animal diseases in a variety of mammalian and avian hosts. While ticks are the accepted vector of piroplasmids, the specific tick vectors for many species remain unknown. Furthermore, prevalence of these pathogens in relation to ecoregions is also largely unknown. This study was conducted to observe the relationship among ecoregions, tick species prevalence, and piroplasm prevalence. Ticks were collected May-August, 2014 from four sites: two in the Blue Ridge Mountain ecoregion and two in the Piedmont ecoregion in Georgia, USA. All sites were selected based on evidence of bobcat activity within the past year in order to optimize conditions for the collection of *Cytauxzoon felis,* which is the most rarely detected of these piroplasms. Polymerase chain reaction (PCR) assays specific to piroplasms were used to test the 263 ticks collected. The three species of ticks studied were *Amblyomma americanum* (n=72), *Dermacentor variabilis* (n=104), and *Amblyomma maculatum* (n=87). The prevalence of both *Amblomma* spp. was higher in the Piedmont, while *D. variabilis* prevalence did not vary between ecoregions (Fisher's exact test, $p<0.0001$). Piroplasms were detected in 15 (5.7%) ticks, with *Theileria* spp. in six (2.3%), *Cytauxzoon* spp. in zero (0%), and *Babesia* spp. in two (0.8%). In addition, an unknown organism was detected in seven (2.7%) ticks, which may be a novel piroplasmid. No difference in piroplasm prevalence between ecoregions was detected (Fisher's exact test, $p=0.2071$). To my knowledge, this report of *Babesia* in *A. maculatum* is the first documentation of any piroplasmid in this tick species.

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Acknowledgements

This study was generously funded by the Dr. Edgar and Mrs. Frances L. Turner Research Grants, the James G. Lester Endowment, and the Scholarly Inquiry and Research at Emory Program. I thank my advisor, Thomas Gillespie, and my committee members, Uriel Kitron and Jaap De Roode, for their support during this study. I am also grateful to Amanda Jo Newkirk-Williams for her assistance with statistical analyses, laboratory work, and field collections. I also thank Gregory Dasch (Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention) for providing additional supplies and the Division of Parasitology (Centers for Disease Control and Prevention) for graciously providing the positive control DNA used in this study. Charlie Muise, Russell Wright, Liz Hornsby, and the Clayton County Water Authority also kindly provided access to my field collection sites. I also thank Sonia Dattaray, Zana Pouncey, Elliyah Dossantos, Amelia Bagwell, and Sarina Hensley for assisting with field collections.

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INTRODUCTION

Ticks are responsible for transmitting a greater diversity of pathogens to both humans and animals worldwide than any other vectors (Jongejan and Uilenberg, 2004). Ticks are best known for their role as vectors of bacteria and viruses, but they also play a key role in the transmission of many protozoa. Apicomplexan protozoa of the order Piroplasmida are a notable example of tick-borne protozoa because they can cause disease in both humans and animals. However, the study of ticks as vectors of piroplasms has been largely neglected (Florin-Christensen and Schnittger, 2009).

The three major genera of piroplasmids are *Babesia*, *Cytauxzoon*, and *Theileria*. Ticks are the only known vector of these genera. Most piroplasmids are spread exclusively by horizontal transmission between their tick vector and vertebrate hosts, but some species, such as *Babesia bovis*, are also capable of vertical transmission within ticks. Vertical transmission occurs when the piroplasm invades tick eggs and develops to its infectious stage in the salivary glands of the progeny (Florin-Christensen and Schnittger, 2009). *Babesia* spp. are known to cause diseases in a wide range of hosts. Animal hosts include mammals, such as ungulates and canines (Criado et al., 2006), and avian species (Peirce and Parsons, 2012). The species *Babesia microti* is also of great concern because it is the agent of human babesiosis (Hersh et al., 2012). *Rhipicephalus microplus* (Riek, 1964) and *Ixodes* spp. (Mather et al., 1990; Randolph, 1991) have been documented as vectors of *Babesia* spp. Recently, *Babesia* spp. were also found in *Amblyomma americanum*, which marks the first documentation of *Babesia* spp. in *Amblyomma* spp. in the New World (Shock et al., 2014).

Cytauxzoon spp. have not been as highly researched as *Babesia* spp., and most existing literature is not focused on the tick vector. *Cytauxzoon felis* is the most well-known species

because it is the agent of the disease cytauxzoonosis, for which the bobcat (*Lynx rufus*) is the only known reservoir (Kier et al., 1987). Cytauxzoonosis has high mortality rates primarily in domestic cats (Meinkoth et al., 2000), but fatal cases have also been documented in tigers (*Panthera tigris*) (Garner et al., 1996; Jakob and Wesemeier, 1996), lions (*Panthera leo*) (Peixoto et al., 2007), and in bobcats (*L. rufus*) (Nietfeld and Pollock, 2002). The reported prevalence of *Cytauxzoon felis* is low (1.9% infection rate or less) in both field-collected *A. americanum* (Reichard et al., 2010) and *Dermacentor variabilis* (Shock et al., 2014).

Theileria spp. have only been documented in ungulates, including bovine, deer, and horse species (Chae et al., 1999; Stockham et al., 2000; Yabsley et al., 2005; Scoles et al., 2011). Like *Cytauxzoon* spp., *Theileria* spp. infections have never been documented in humans, but fatal *Theileria* spp. infections have been reported from bovines and equines (Chae et al., 1999; Scoles et al., 2011). Documented tick vectors for *Theileria* spp. include *A. americanum* (Reichard and Kocan, 2006), *Amblyomma cajennense* (Scoles et al., 2011), and *D. variabilis* (Chae et al., 1999).

While some studies have examined differences in piroplasm prevalence in ticks from different geographic regions (*i.e.* states) (Shock et al., 2011, 2014; Mueller et al., 2013), there has been little attention to variations between ecoregions, which have a direct impact on tick-borne disease ecology. Variations in ecoregions have been shown in other systems to contribute to differences in tick species prevalence (Estrada-Peña et al., 2006; Illoldi-Rangel et al., 2012) and differences in piroplasm prevalence (Moncayo et al., 2010). *Cytauxzoon felis* is especially dependent on ecoregion because its reservoir host range is more limited than that of *Babesia* and *Theileria* spp.

In this study, I addressed multiple questions associated with the piroplasmids *Babesia*, *Cytauxzoon*, and *Theileria* in relation to their tick vectors and ecoregions. I selected active

bobcat territories for tick collection sites to increase the likelihood of encountering *C. felis* in ticks to contribute data on the neglected pathogen, potentially revealing vector species preferences previously obscured by low detection rates in field-collected ticks. In addition, I selected collection sites in two major ecoregions in Georgia (Figure 1) in order to determine if there was a relationship between ecoregions, tick species prevalence, and piroplasmid prevalence. Finally, to my knowledge none of these piroplasmids has ever been documented in *Amblyomma maculatum* ticks, perhaps due to the small sample size tested in previous studies (Shock et al., 2014), so I examined the prevalence of piroplasmids in the largest sample of *A. maculatum* tested to date.

MATERIALS AND METHODS

Collection site identification

Tick collection sites were selected based on a combination of geographic location, with an equal number of collection sites in two ecoregions, and known *L. rufus* activity within the past year in order to increase the likelihood of collecting multiple species of ticks infected with *C. felis*. Sites with *L. rufus* presence were identified through landowners' contributions to a listserve designed to communicate wildlife sightings. Landowners were contacted through the listserve and permission was obtained to collect ticks and to survey the property for signs of bobcat activity. Bobcat activity was confirmed at sites through wildlife trail camera photographs and scat. Four sites were selected for inclusion in this study: two in the northern Blue Ridge Mountain ecoregion and two in the central Piedmont ecoregion to observe variations in vector and pathogen prevalence (Figure 1).

Tick collection and identification

Ticks were collected from May to August 2014 at least once per month, excepting one site in the Piedmont ecoregion where collections only occurred in May due to logistical constraints. Questing ticks (n=140) where collected by running a 1 m^2 flannel cloth over vegetation and by collecting ticks from people and domestic canines prior to attachment (n=123) for a total of 263 samples. Samples were stored in 70% ethanol at 4ºC. Ticks were identified to life stage and species using morphological characteristics (Strickland, 1976; Keirans and Litwak, 1989; Keirans and Durden, 1998).

DNA extraction

Before DNA extraction, ticks were washed sequentially with 10% bleach, 70% ethanol, and three distilled water rinses to reduce surface contamination. Adults were bisected and DNA was extracted from one half of each individual. DNA extraction was performed with the Promega Wizard SV 96 Genomic DNA Purification System (Promega, Madison, Wisconsin). Purified DNAs were stored at 4ºC for the duration of this study.

Polymerase chain reaction

All samples were screened with a polymerase chain reaction (PCR) that amplified the entire internal transcribed spacer region (ITS1) of *Cytauxzoon*, *Babesia*, and *Theileria* spp. using the forward primer ITS-15C (5’ CGATCGAGTGATCCGGTGAATTA 3’) and the reverse primer ITS-13B (5’ GCTGCGTCCTTCATCGTTGTG 3’) (Bostrom et al., 2008). For this primer set, each reaction mix contained 10µL Taq PCR Master Mix (Qiagen, Hilden, Germany), 6µL nuclease-free water, 0.25 µM each forward and reverse primer, and 2µL template DNA for a total reaction volume of 20µL. A nested PCR was performed using the forward primer ITS-15D

(5’ AAGGAAGGAGAAGTCGTAACAAGG 3’) and the reverse primer ITS-13C (5’ TTGTGTGAGCCAAGACATCCA 3’) (Shock et al., 2011). For the nested reaction, each reaction mix contained 10µL Taq PCR Master Mix (Qiagen, Hilden, Germany), 7µL nuclease-free water, 0.25 µM forward and reverse primer, and 1µL template for a total reaction volume of 20µL. Both reactions were performed in a Mastercycler Pro (Eppendorf, Hamburg, Germany) using the following thermocycler conditions: one step of 94ºC (1 min), 35 cycles of 94ºC (30 sec), 52ºC (30 sec), 72ºC (1 min), and one step of 72ºC (5 min). A positive control reaction with template DNA from a human clinical patient blood sample infected with *B. microti* was included with each set of reactions. The positive control DNA was kindly provided by the Division of Parasitology at the Centers for Disease Control and Prevention and was extracted using the Qiagen DNeasy Kit (Qiagen, Hilden, Germany). Amplicons were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide.

Sequencing

The amplicons were visualized on a 1% agarose gel, and bands >200bp (Shock et al., 2014) were excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Purified amplicons were sequenced bi-directionally using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an ABI 3130xl Genetic Analyzer. Sequences were aligned in Geneious (version 7.1.3) (http://www.geneious.com, Kearse et al., 2012) and compared to existing sequences in GenBank by BLAST.

Data Analysis

All data analysis was performed using R statistical platform (version 3.1.2) (R Core Team, 2013). Fisher's exact tests (Agresti, 2002) were used to determine the relationship between tick species and ecoregion, between piroplasm prevalence and ecoregion, and between tick species and piroplasmid infection. A Mantel-Haenszel chi-squared test (Agresti, 2002) was used to determine if there was a relationship between collection date and piroplasmid infection while controlling for tick species. Pairwise Fisher's exact tests were used for post hoc analysis. Holm's correction (Holm, 1979) for multiple comparisons was applied where appropriate.

RESULTS

Seventy-two *A. americanum* (27.4%, n=263), 87 *A. maculatum* (33.1%, n=263), and 104 *D. variabilis* (39.5%, n=263) were collected from May-August 2014 (Figure 2) and assayed for piroplasmids (Table 1). When analyzing the relationship between ecoregion and tick species abundance, a Fisher's exact test found a difference in species abundances between ecoregions (p<0.0001). Post hoc tests found no difference in the geographic distribution of *A. americanum* and *A. maculatum* between ecoregions (corrected p=0.45); both species were found almost exclusively in the Piedmont (Table 1). However, *D. variabilis* was found in similar numbers in both ecoregions, differing from both *A. americanum* (corrected p<0.0001) and *A. maculatum* (corrected p<0.0001) (Table 1).

A total of 15 ticks were PCR-positive for piroplasms (5.7%), of which two ticks were *Babesia* spp. positive (0.8%), six were *Theileria* spp. positive (2.3%), and seven were positive for an unknown organism (2.7%) (Figure 3, Table 2). No *C. felis* positive ticks were detected. Fourteen PCR-positive ticks were collected in the Piedmont ecoregion, and only one was

collected in the Blue Ridge ecoregion. However, a Fisher's exact test found no difference in piroplasm prevalence between ecoregions (p=0.2071). *Theileria* spp. were only found in *A. americanum* (16.7%) (Figure 3, Table 2), a significantly higher prevalence compared to the other tick species (Fisher's exact test, p=0.008). There was no difference in the relationships between the unknown organism and tick species (all pairwise tests corrected p>0.05). A single *Babesia* positive sample was found among the *A. americanum* and *A. maculatum* tested (Figure 3, Table 2). No statistical analyses were performed for *Babesia* spp. infection due to an insufficient sample size. The effect of date on *Theileria* spp. infection when controlling for species was also analyzed for the months of May and June, but July and August were excluded due to smaller sample sizes. No effect was detected for May (Mantel-Haenszel chi-squared, p=0.1979) or June (Mantel-Haenszel chi-squared, p=0.2512).

Sequences were generated for 10 out of the 15 PCR-positive piroplasms (Table 3). Two of the sequences were 99% identical to *A. americanum*-derived *Theileria* sequences in GenBank (Table 3). Four other PCR-positive tick DNAs were also identified as containing *Theileria* based on similarity in band size to the products verified by sequencing (Figure 4). Two sequences were also identified as *Babesia* spp. BLAST analysis of these *Babesia* spp. ITS1 sequences revealed a 95-99% identity to *Babesia* sequences from an *A. americanum* tick (KC119624) and from a *D. variabilis* tick (KC162895) (Table 3). No sequences generated in this study were consistent with *Cytauxzoon* spp. sequences in GenBank.

Six of the sequences generated in this study did not show significant identity to any existing sequences in GenBank. The closest match for all six sequences was the same uncultured soil fungus ITS1 region (Table 3), but query cover was too low (27-51%) to identify these sequences as originating from fungi. All sequences were of high quality (HQ% 95-100%), with

high identity between sequences generated from different ticks. Both *D. variabilis* ticks positive for a piroplasmid generated unknown sequences, and they are identical to each other and to one *A. americanum* sequence (Figure 5). Two other of these unknown ITS1 PCR-positive sequences in *A. americanum* were also identical to each other (Figure 5). Based on band size, one unsequenced PCR-positive was also identified as a similar unknown (Figure 4).

DISCUSSION

Piroplasms are a rising health concern for animals and humans, with each *Babesia*, *Cytauxzoon*, and *Theileria* spp. parasitizing a unique range of hosts. However, the study of piroplasms and their relationship to their tick vectors has been neglected; the vectors and reservoirs of many piroplasms remain unknown (Chae et al., 1999; Florin-Christensen and Schnittger, 2009; Hersh et al., 2012). I conducted this study to determine the prevalence of piroplasms in questing ticks collected from active bobcat territories in distinct ecoregions. By selecting active bobcat territories, I increased the potential to collect *C. felis* infected ticks to provide data on which ticks played a major role in the transmission of the pathogen. In addition, I collected ticks from two major ecoregions in the state of Georgia in order to increase the diversity of tick species collected, which allowed me to also observe the relationship between geography and tick species diversity.

In my study, there was a significant difference in the abundance of tick species between the ecoregions, with nearly all *Amblyomma* spp. ticks being collected in the Piedmont ecoregion and *D. variabilis* being found equally in both regions. Finding that *A. americanum* and *A. maculatum* were common in the Piedmont ecoregion is consistent with previous literature (Mixson et al., 2006) . However, there is a lack of literature available on variation in *D.*

variabilis prevalence between ecoregions, and therefore it is not clear if their distribution is consistent with historic distributions or if it represents a change. Further research expanding into more ecoregions is necessary to better understand tick species geographic distribution.

I also focused my collection sites to active bobcat territories in order to study the prevalence of *C. felis* and its vectors. To my knowledge there has been no documentation of vertical transmission of *Cytauxzoon* spp. I therefore focused testing on adult ticks, which have a higher probability of piroplasmid infection than immature life stages because they have taken more bloodmeals. However, I did not find any *C. felis* positive ticks. This could be an indication that *C. felis* prevalence is extremely low, even in areas where a potential reservoir host is knownto occur. The existing literature has never documented a prevalence higher than 1.9% in any tick vector (Reichard et al., 2010; Shock et al., 2014), so even the highest estimation of prevalence would require at least a sample size of *n=157* for a 95% chance of finding one positive tick. Because I did not have this large of a sample size for any one tick species, it is possible that *C. felis* was present at the collection sites but not detected in this study. In addition, very few ticks have been documented as *C. felis* positive in Georgia prior to this study (Shock et al., 2014). Furthermore, a study of prevalence of *C. felis* in bobcats found that only 9% of bobcats in Georgia were *C. felis* positive, compared to the highest prevalence of 79% in Missouri (Shock et al., 2011). Therefore, the lack of any *Cytauxzoon* spp. in ticks is consistent with the low prevalence of *C. felis* in Georgia.

To my knowledge, this is the first documented case of *Babesia*-infected *A. maculatum*, which has never been documented as a vector for any piroplasm (Shock et al., 2014). An earlier study of piroplasms in various tick vectors only tested 16 *A. maculatum* ticks for piroplasmids, so this discovery could be largely attributed to the a larger *A. maculatum* sample size (*n*=87) in

the present study. The *Babesia* spp. found in *A. maculatum* in this study was 99% similar to a *Babesia* sp. detected in a *D. variabilis* tick (Table 3). It is not known whether this *Babesia* sp. is a human or veterinary pathogen; I am also not able to determine from this study if *A. maculatum* is a viable transmission vector for *Babesia* or if the piroplasmid detected was viable. Additional research is necessary to evaluate the role *A. maculatum* as a vector for *Babesia* spp. as well as other piroplasmids. Furthermore, this is also the second study to document *Babesia*-infection in any *Amblyomma* spp. (Shock et al., 2014), and my study confirms *A. americanum* as a potential vector for *Babesia* spp.

In this study, I also found a variety of unknown ITS1 PCR-positive sequences. Although these sequences do not match any existing sequences in GenBank, the high quality of the sequences and the conservation between themselves indicates that these are valid findings. These sequences were isolated from ticks that were collected in two different ecoregions across multiple different time periods. In addition, the sequences were from different tick species, including males, females, and nymphs. DNA from these ticks was also extracted at separate times. Template-free negative controls were included in each PCR assay and no indication of contamination was detected. These sequences could be a previously undocumented piroplasm or entomopathogenic fungus, but it is not clear from the limited sequence data available. This is because the ITS1 spacer region is ubiquitous across eukaryotes, and there is an ITS1 region in a different locus in some bacteria and archaea (Rogers, 2011). However, the nested primers used in this paper were analyzed with BLAST, and though some species of fungi could potentially bind with the forward primer, the reverse primer was highly specific to piroplasmids. Additional sequence data targeting other gene regions will be necessary to identify the source of these unknown sequences.

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FIGURES

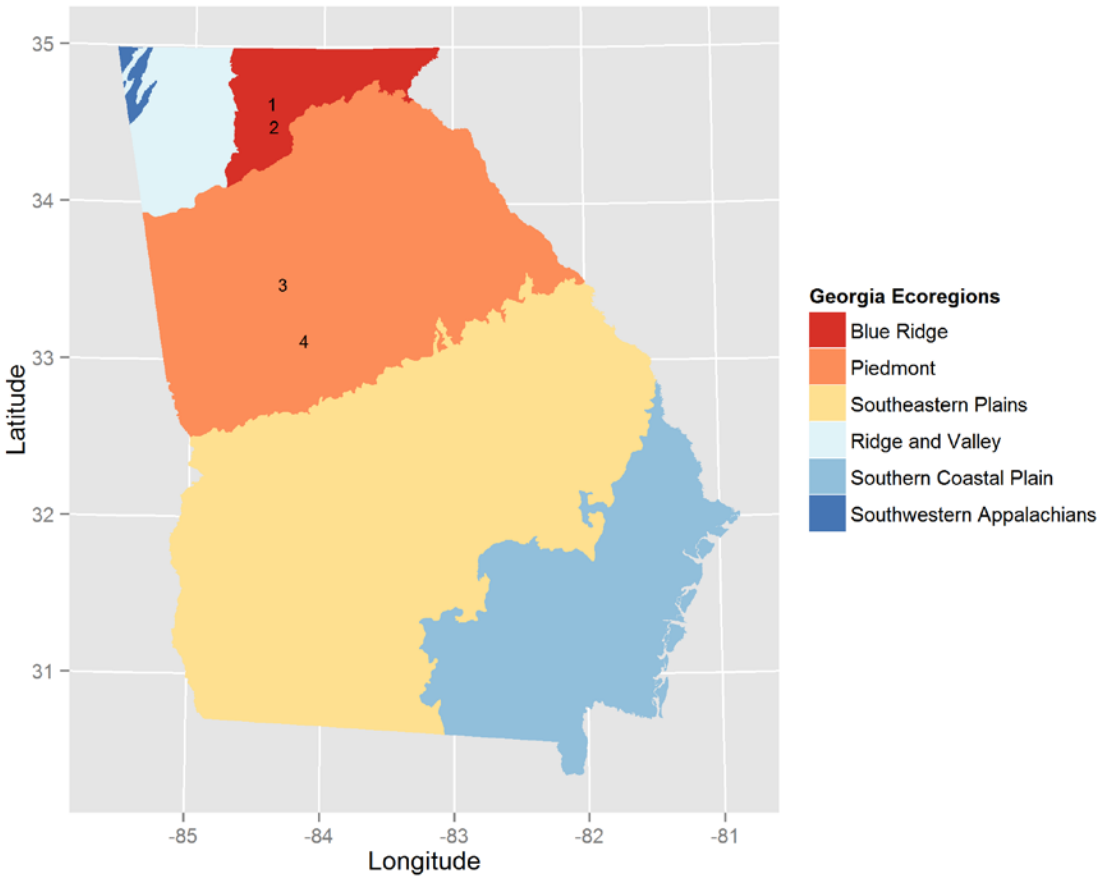

Figure 1. Tick sample site locations within Georgia ecoregions. Sample sites are indicated by numbers on the map; see Table 1 for additional information on ticks collected from each site.

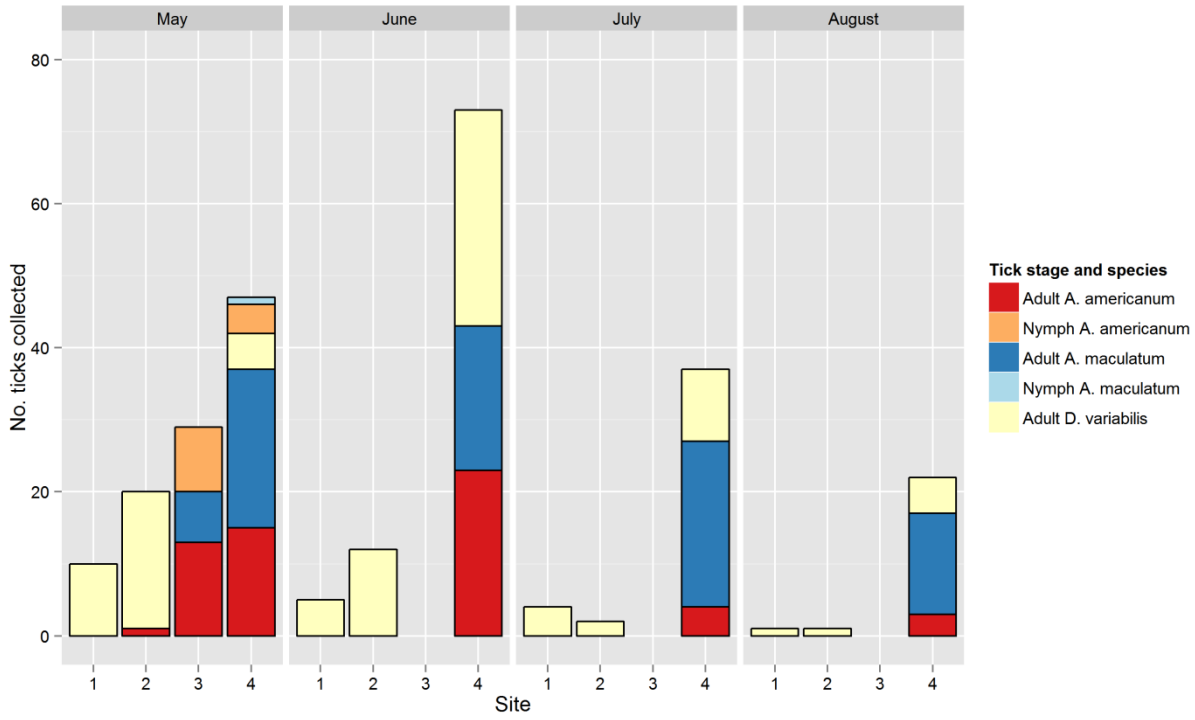

Figure 2. Number of ticks tested May-August 2014 from four collection sites with known bobcat activity in the past year, which were tested for piroplasmids. 1 and 2 represent the two Blue Ridge ecoregion sites, and 3 and 4 represent the two Piedmont ecoregion sites.
Note that this is not an accurate reflection of tick species and life stage prevalences at each site because I focused on adult ticks when testing for piroplasmids.

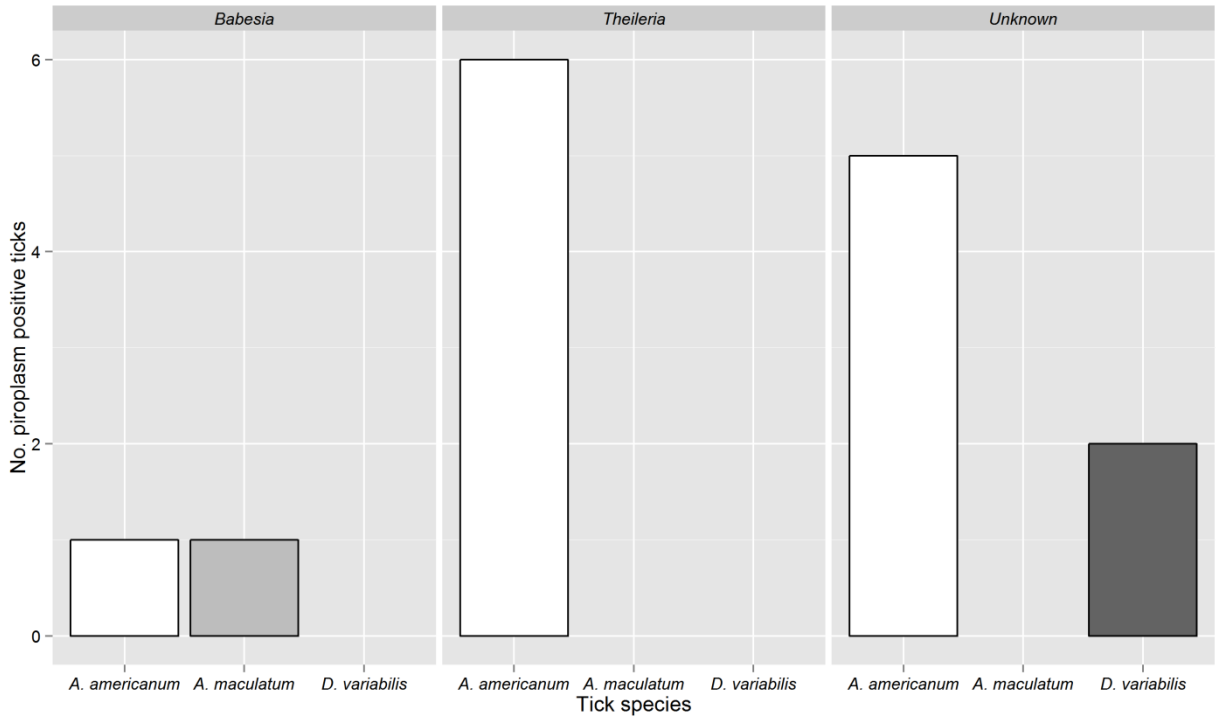

Figure 3. Number of piroplasm-positive tick samples by piroplasm genera and tick species.

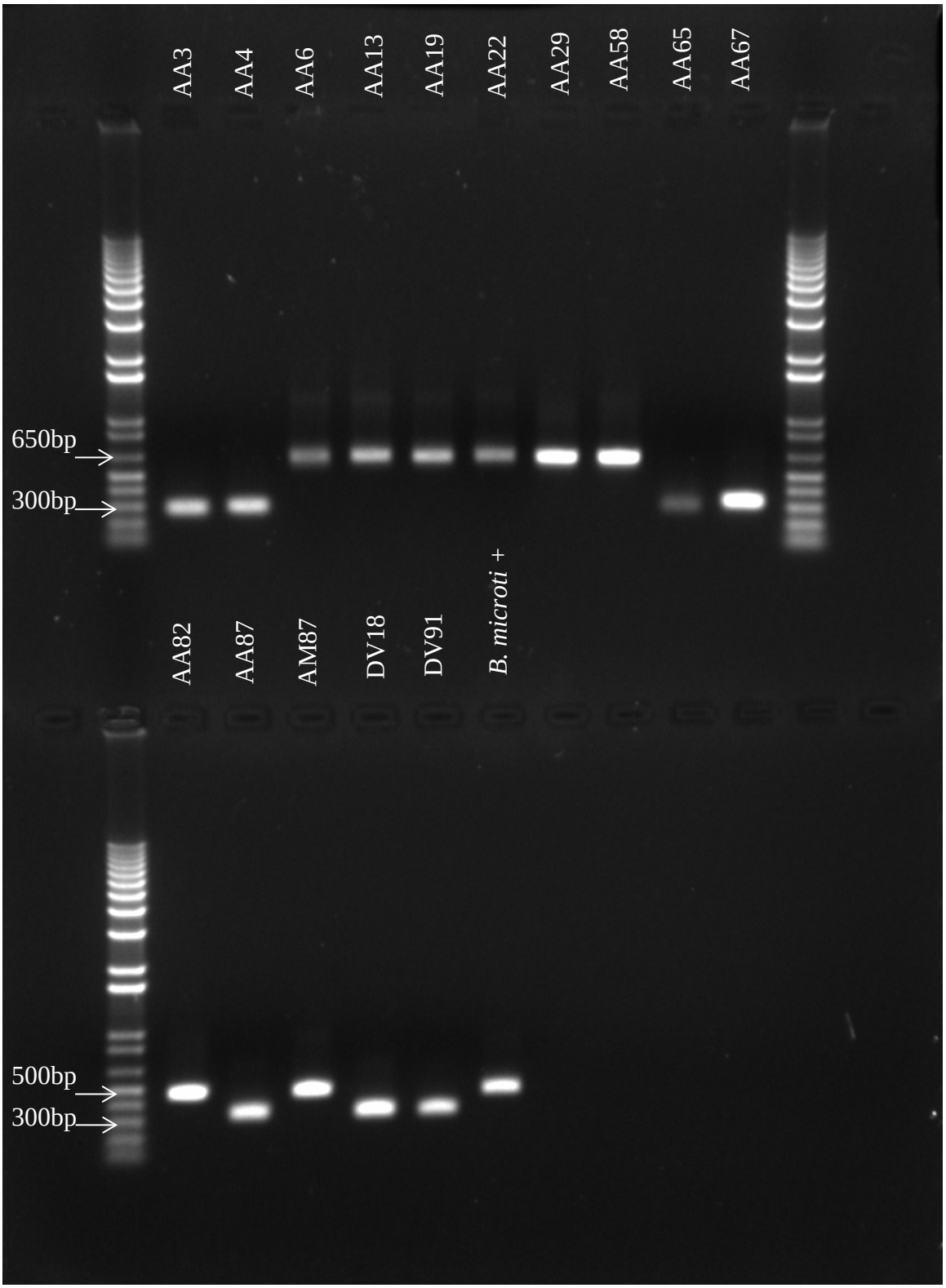

Figure 4. Gel electrophoresis image of the purified nested ITS1 amplification products from piroplasmid-positive ticks. Alpha-numerical sample identifiers are given for each lane. Relevant standard band sizes are labeled on the left of the image.

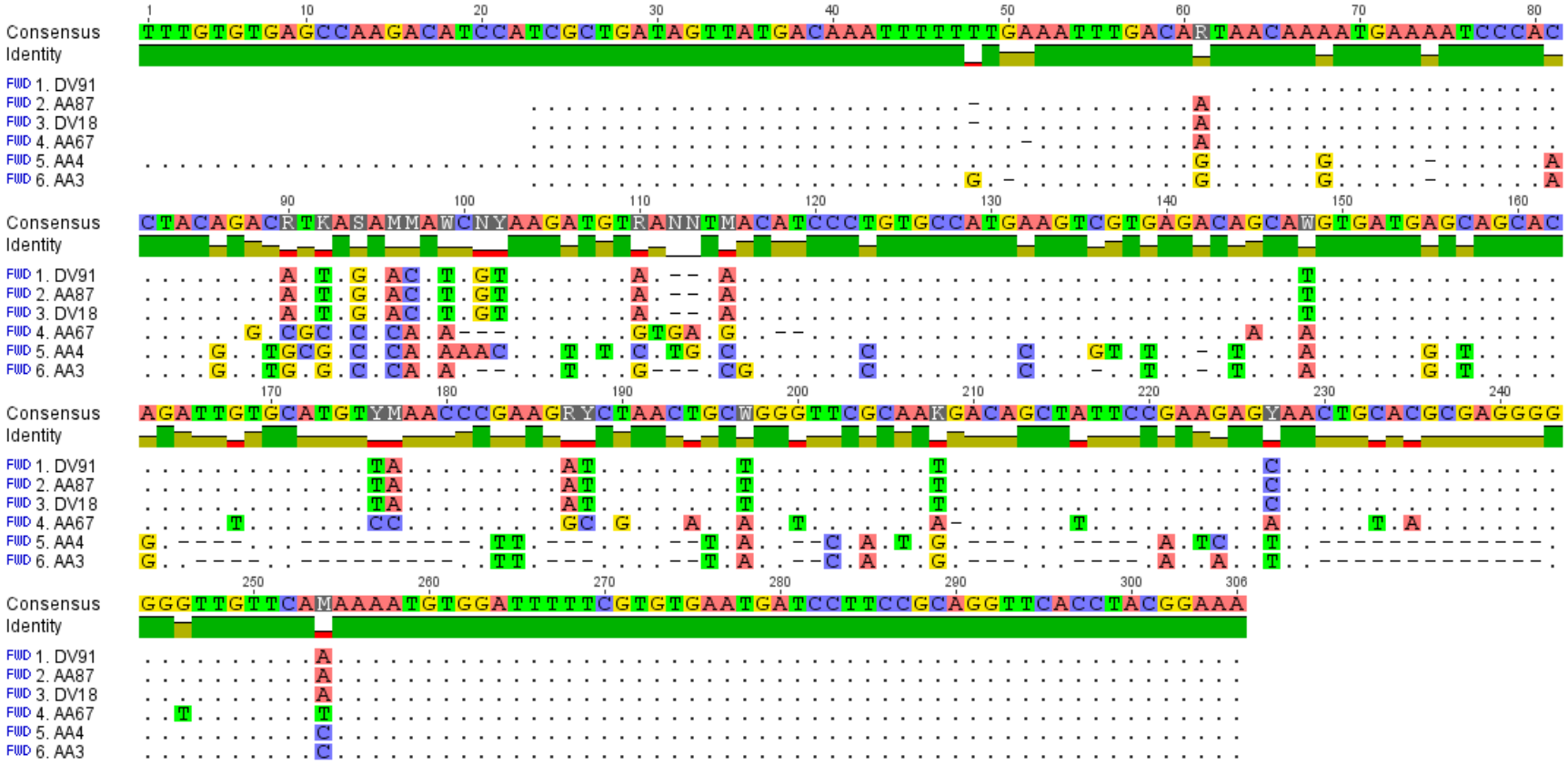

Figure 5. Alignment of unknown sequences from tick DNA tested with the ITS1 nested primers. The consensus sequence is given at the top of each row; agreement to the consensus is indicated by a dot.

TABLES

Table 1. Tick samples collected from each ecoregion in Georgia.

	Blue Ridge (North)		Piedmont (Central)	
Tick Species	1	2	3	4
*Amblyomma americanum**	0	1	22	49
*Amblyomma maculatum**	0	0	7	80
Dermacentor variabilis	20	34	0	50
Totals	20	35	29	179

*Difference between ecoregions significant (p<0.0001)

Table 2. Prevalence of piroplasmids detected in ixodid ticks from active bobcat territories.

Tick species	No. of females	No. of males	No. of nymphs	*n*	Number positive (%)			
					Babesia spp.	*Cytauxzoon* spp.	*Theileria* spp.	Unknown
Amblyomma americanum	28	31	13	72	1 (1.4)	0	6 (8.3)	5 (6.9)
Amblyomma maculatum	53	33	1	87	1 (1.1)	0	0	0
Dermacentor variabilis	47	57	0	104	0	0	0	2 (1.9)
Totals	128	121	14	263	2 (0.8)	0	6 (2.3)	7 (2.7)

Table 3. Sequence analysis results and identification of piroplasmids in ticks collected from active bobcat territories.

Tick species	Tick ID	Piroplasm	Closest match in GenBank			
			Accession no.	% identity	% query cover	Base pairs of ITS1 sequence
A. americanum	2014Aamer3	Unknown	EU490149, Uncultured soil fungus	97	51	235
A. americanum	2014Aamer4	Unknown	EU490149, Uncultured soil fungus	97	54	263
A. americanum	2014Aamer19	*Theileria spp.*	KC119627, *Theileria sp.*	99	100	530
A. americanum	2014Aamer58	*Theileria spp.*	KC122669, *Theileria sp.*	99	100	347
A. americanum	2014Aamer67	Unknown	EU490149, Uncultured soil fungus	96	41	277
A. americanum	2014Aamer82	*Babesia spp.*	KC119624, *Babesia sp.*	95	100	424
A. americanum	2014Aamer87	Unknown	EU490149, Uncultured soil fungus	96	41	281
A. maculatum	2014Amac87	*Babesia spp.*	KC162895, *Babesia sp.*	99	100	395
D. variabilis	2014Dvar18	Unknown	EU490149, Uncultured soil fungus	96	41	281
D. variabilis	2014Dvar91	Unknown	EU490149, Uncultured soil fungus	96	27	241
A. americanum	2014Aamer6	*Theileria spp.*	NS			
A. americanum	2014Aamer13	*Theileria spp.*	NS			
A. americanum	2014Aamer22	*Theileria spp.*	NS			
A. americanum	2014Aamer29	*Theileria spp.*	NS			
A. americanum	2014Aamer65	Unknown	NS			

NS = not sequenced