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Molecular Differentiation of Metastriate Tick Immatures

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Abstract

Hard ticks, family Ixodidae, are divided into two groups, the Metastriata and the Prostriaata, based on morphological differences. In the United States, there are four medically important genera of the Ixodidae: *Ixodes*, *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. *Ixodes* is the only genus in and representative of the Prostriaata, whereas the latter three genera are members of the Metastriata. All developmental stages of the Prostriaata can be easily differentiated from the Metastriata using morphology. Similarly, the three Metastriate genera are highly identifiable as adults, yet as immatures, the discriminating characteristics can be difficult to use for differentiation, especially if the specimens are damaged or engorged with blood. All three Metastriate genera represent medically important vectors, thus accurate differentiation is necessary. To this end, we have developed a multiplexed-PCR diagnostic assay that, when combined with RFLP analysis will differentiate between the Metastriate genera—*Amblyomma*, *Dermacentor*, *Rhipicephalus*, and *Haemaphysalis* based on the length of the PCR amplicon and subsequent restriction digestion profile. The intended use for this diagnostic is to verify morphological identifications, especially of immatures, as well as to identify samples destroyed for molecular analysis, which will lead to more accurate field data as well as implication of vectors in disease transmission.

Keywords

Metastriate; *Amblyomma*; *Dermacentor*; *Haemaphysalis*; *Rhipicephalus*; diagnostic

Introduction

THE FAMILY IXODIDAE, or hard ticks (Arachnida: Acari: Parasitiformes) consists of 694 species divided into two main morphological and phylogenetic groups: the Prostriaata and the Metastriata (Sonenshine 1991). The Prostriaata are represented by the single subfamily Ixodinae that consists of one genus, *Ixodes*. In contrast, the Metastriata consists of five subfamilies; *Amblyomminae*, *Haemaphysalinae*, *Hyalomminae*, *Rhipicephalinae*, *Bothriocrotoninae* (Black and Piesman 1994, Klompen et al. 2002). Of the thirteen described genera in the Metastriata, seven have species that are implicated in disease transmission (Hoogstraal and Wassef 1986). All Ixodidae are hematophagous and obligate ectoparasites.

Medically important species are represented in several genera of hard ticks found in the United States, including *Ixodes*, *Amblyomma*, *Dermacentor*, *Rhipicephalus* and *Haemaphysalis* (Walker 1998). Of these genera, *Ixodes* is the largest, with more than 200 recognized species. *Ixodes scapularis*, the black-legged tick, is probably the most recognized species of this genus and serves as the vector of Lyme disease, human babesiosis, granulocytic ehrlichiosis and Powassan encephalitis virus in North America.

The most frequently encountered representative of the genus *Dermacentor* in the eastern United States is *D. variabilis*, the American dog tick. This species is implicated in the transmission of numerous species of *Rickettsia*, including the etiologic agent of Rocky Mountain Spotted Fever (RMSF) and can cause tick paralysis. *Dermacentor andersoni*, the Rocky Mountain wood tick, as its name suggests is not common on the east coast, and is primarily found in the western United States. This tick has been implicated as the vector of the agents that cause RMSF, Colorado tick fever, bovine anaplasmosis and Tularemia (Walker 1998). The Pacific coast tick, *D. occidentalis*, is restricted to the Pacific lowlands from Oregon to Baja California and is a natural vector for *Anaplasma marginale* Theiler. Unlike the previously mentioned *Dermacentor* species, the winter tick, *D. albipictus*, is a one host tick, completing its life cycle on white tailed deer (*Odocoileus virginianus*) and other large ungulates. While common in the Mid-Atlantic region *D. albipictus* is not associated with human disease transmission, but does vector bovine anaplasmosis (Stiller et al. 1981).

The most common *Amblyomma* tick in the eastern and mid-Atlantic United States is *Amblyomma americanum*, the Lone Star tick, which has been reported to vector *Ehrlichia chaffeensis* (Dumler and Bakken 1998) and the pathogens that cause tularemia and Q fever (Parola and Raoult 2001). Another *Amblyomma* species, *A. maculatum*, the Gulf Coast tick, is one of several vectors of *Cowdria ruminantium* Cowdry, the causative agent of the veterinary illness, heartwater, and is usually found on large animals (Uilenburg 1982). This tick is primarily found along the southern coastline between the Atlantic and Gulf Coast and is only occasionally found in more inland locations (Bishop and Hixson 1936, Harrison et al. 1997). Although considered a serious economic pest, no known human disease transmission is associated with *A. maculatum*.

The brown dog tick, *Rhipicephalus sanguineus*, is not as common but is occasionally found in the mid-Atlantic region and can vector canine babesia, Texas cattle fever, and canine hepatozoonosis (Ewing et al. 2000). *Haemaphysalis leporispalustris*, the rabbit tick, as its name suggests, primarily feeds on rabbits and birds and is not usually anthropophilic. While it has been speculated that this tick may vector several agents and serve as a potential bridge vector for RMSF, it does not appear to be a major vector for human pathogens (Goethert and Telford 2003).

Morphologically, these five tick genera are easily identifiable as adults. In the nymphal and larval stages the anal groove, the cuticular fold found near the anal aperture, clearly differentiates the Prostriata, with the groove anterior to the anus, from the Metastriata, on which the anal groove is either posterior to the anal aperture or is absent. Among the Metastriate, differentiation of nymphal and larval ticks has proven more difficult, especially

if partially or fully engorged, or if the specimen is damaged or morphologically incomplete. A common dichotomous key used for the identification of ticks found in the northeast United States (Sonenshine 1979) is a useful guide for distinguishing hard ticks. However, many distinguishing characteristics are difficult to locate or identify in immature specimens, especially by those untrained in acarine taxonomy. For those unfamiliar with the taxa, the Metastriate immatures become especially difficult to differentiate and may be mis-identified. This problem is often compounded during molecular surveys for pathogens or genetic analysis, as the entire immature specimens are often destroyed. Therefore, an alternative method for rapid and accurate identification of Metastriate immatures is needed, especially for verification of specimen identification post-processing.

Mitochondrial and nuclear DNA has been used extensively to conduct phylogenetic studies (Black and Piesman 1994, Crampton et al. 1996, Black et al. 1997, Klompen et al. 1996, Norris et al. 1997, Dobson and Barker 1999), to check the validity of species designations (Wesson et al. 1993, Caporale et al. 1995, Norris et al. 1997), and in population genetic studies of Ixodid ticks (Rich et al. 1995, Norris et al. 1996). In each of these studies, PCR has been employed using specifically designed primer sets (Sparagano et al. 1999). The PCR product is then sequenced or screened using techniques such as single-strand confirmation polymorphism (SSCP) analysis, and nucleotide insertion, deletion or point mutations are then used to distinguish one species from another (Hiss et al. 1994, Qiu et al. 2002). These procedures can be both time consuming and costly. This study describes a novel PCR-RFLP based assay to clearly differentiate between the four most common Metastriate tick genera in the United States: *Dermacentor*, *Amblyomma*, *Rhipicephalus*, and *Haemaphysalis*.

Methods and Materials

Tick material

Ixodes scapularis, *D. variabilis*, and *A. americanum* adults were collected by standard flagging techniques. Collections were made during the spring, summer and fall of 2001 at various locations in central and southern Maryland. Voucher specimens of *I. scapularis*, *D. variabilis*, *R. sanguineus*, and *A. americanum* nymphs and larvae, and adult *D. occidentalis* were kindly provided from colonies maintained at the Centers for Disease Control and Prevention (Atlanta, GA). Voucher specimens of adult *D. andersoni*, larval *D. albipictus*, and adult *A. americanum* were graciously provided by G. Scoles (USDA, Washington State University, Pullman, WA). Specimens representing *R. sanguineus*, *A. maculatum*, and *H. leporispalustris* were purchased from the tick rearing facility at Oklahoma State University (Stillwater, OK). Several archived specimens of adult and nymph *D. albopictus* were provided by J.R. Keirans (Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, GA).

PCR primer design and RFLP analysis

External primers employed in this study have been previously described (Black and Piesman 1994, Norris et al. 1996). Novel, internal, genus-specific primers were designed using a representative sample of previously published 16S sequences from GenBank for representative species of *Dermacentor*, *Amblyomma*, *Rhipicephalus*, *Haemaphysalis* and

Ixodes tick (Table 1). The sequences were aligned using a multiple pairwise alignment program (MegAlign, DNASTar, Madison, WI). Analysis of the alignment revealed a 24-bp region which appeared to differentiate the major genera (Table 1). Two internal primers, 16sDvF2 and 16sAmR, were designed to amplify *Dermacentor* and *Amblyomma* species, respectively (Table 2, Fig. 1). The internal primers were manually selected with the assistance of Primer 3 (Rozen and Skaletsky 2000) to calculate T_m and primer compatibility.

To differentiate *Rhipicephalus* from *Haemaphysalis* ticks, restriction enzyme recognition sites along the mitochondrial 16S gene were mapped using a world wide web based Restriction Mapper program (www.restrictionmapper.org). Using the previously described 16S ribosomal DNA alignment of various tick species, only enzymes which would cut either *Rhipicephalus* or *Haemaphysalis*, but not both, were chosen for further evaluation.

DNA extraction and amplification

Adult ticks were surface sterilized and bisected medially with a sterile scalpel, maintaining one half at -80°C as a voucher and utilizing the other half for genomic DNA extraction. DNA was extracted from ticks by modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction (Johnson et al. 1992, Black et al. 1997). The described CTAB homogenization was followed by a phenol/chloroform/isoamyl alcohol (25:24:1) extraction, a chloroform/isoamyl alcohol (24:1) extraction, isopropanol precipitation, and resuspension in 50 μL of HPLC grade H_2O or $0.5 \times \text{TE}$ buffer. Nymphal and larval ticks were extracted utilizing the same methods but using the entire specimen and were resuspended in 30 μL of HPLC H_2O or $0.5 \times \text{TE}$ buffer. All extractions were held at -20°C when not in use. Multiplexed PCR was performed using the four primers described in Table 2. The 50- μL reaction mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 1.5 mM dNTPs; 25 μM of primers 16S+1 and 16S-1, 75 μM 16SDvR and 50 μM 16SAmF, 1.5U of *Taq* DNA polymerase, and 2 μL (≈ 30 ng) of DNA template. Differential priming efficiencies were noted among the different primer sets and combinations. Final primer concentrations were optimized by empirical experimentation to arrive at primer combinations that produced approximately equivalent band intensities. PCR amplification was performed by denaturing the samples at 94°C for 5 min, then 35 cycles of 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min with a final extension step at 72°C for 10 min. All amplifications were performed on a MJR PTC-200 96-well thermocycler (MJ Research, Watertown, MA). Amplicons were visualized on 2% agarose gels stained with ethidium bromide.

Amplified products that contained only one band of approximately 460 bp (presumably *Haemaphysalis* or *Rhipicephalus*) were subjected to RFLP analysis using *TauI* restriction enzyme (Fermentas Life Sciences, Hanover, MD) following the manufacturer's instructions. Products of the digestion reaction were visualized on 2% agarose gels stained with ethidium bromide.

Validation of multiplex primer design

Following optimization, the multiplexed primer cocktail was evaluated using 160 nymphs collected from several locations in Maryland. Using a dissecting microscope, all specimens

were tentatively identified as *Amblyomma americanum* when compared to the morphology-based Sonenshine key for ticks in Virginia (Sonenshine 1979). The ticks were then crushed whole and processed for genomic amplification, as described above. As an additional validation, ten colony reared ticks representing various stages of *D. variabilis*, *A. americanum*, *D. andersoni*, and *I. scapularis* were extracted and tested in a manner such that each template was blinded. The investigator tested each template and identified the genus based on the PCR amplification profile. The template identity was revealed and the diagnostic accuracy was determined.

Results

During optimization, all primer combinations produced amplicons of the expected length. Using the four primer multiplexed cocktail, each PCR reaction mixture produced one or two products, the genus-specific amplicon and/or the full length (460 bp) 16S+1/-1 amplicon. Amplicons unique for each tick genus were identified from single PCR reactions; ~306-bp and 460-bp products for *A. americanum*, ~190-bp and 460-bp products for all *Dermacentor* species, or only the fulllength product (460-bp) for *Rhipicephalus*, *Haemaphysalis*, and *Ixodes*. We were unable to derive an amplicon from *A. maculatum* using the internal genus specific primer. PCR products were successfully produced from each DNA template as expected, regardless of life stage utilized for source DNA (Fig. 2).

Rhipicephalus, *Haemaphysalis*, and *Ixodes* (for comparison) amplification products (one band at ~460 bp) were subjected to RFLP analysis using the restriction enzyme *TauI*. *TauI* cuts at the recognition sequence GC[C/G]GC which is present in the mitochondrial 16S rDNA gene fragment of *Rhipicephalus* species but not in *Haemaphysalis* or any of the other species of ticks investigated (Fig. 3). Complete digestion produced a doublet band of ~230 bp (Fig. 4).

Following optimization, the multiplexed primer cocktail was evaluated on 160 nymphs collected from several locations in Maryland. All specimens had been tentatively identified as *Amblyomma americanum* using the morphology-based Sonenshine key for ticks in Virginia (Sonenshine 1979). The PCR-based assay confirmed the *Amblyomma* identification for all specimens. Correct identification of voucher specimens used in the blinded analysis further validated the specificity of the multiplexed PCR-RFLP analysis.

Discussion

Molecular evaluation of ticks for disease transmission and phylogenetic studies has increased tremendously over the past ten years (Sparagano et al. 1999). A considerable amount of this research is being carried out by personnel with little or no training in acarine taxonomy and morphology. Mis-identification can easily lead to erroneous results and, in the case of pathogen transmission, could implicate the incorrect vector species. Additionally, once the specimen is destroyed during DNA extraction, anomalous results are difficult to verify. Although voucher specimens should always be retained and identification verified by qualified taxonomists, the need to validate the identification of extracted tick DNA is crucial. Using established molecular markers for PCR amplification and sequencing can be

extremely cost prohibitive, especially for population studies or surveys where possibly hundreds of samples are processed.

In the mid-Atlantic and northeastern regions of the United States, the main tick genera commonly encountered—*Ixodes*, *Dermacentor*, *Amblyomma*, *Haemaphysalis*, and *Rhipicephalus*—have overlapping seasonal activity and distributions, and most importantly have the potential to pose as threats to public health. Subsequently, tick collections often consist of more than one tick species at any given time. Misidentification of tick samples could lead to inaccurate disease transmission rates, incorrect host association as well as misinterpretation of geographic and habitat range.

Three of the tick species used in this study—*D. variabilis*, *A. americanum*, and *I. scapularis*—are predominant ticks commonly encountered in Maryland and are all medically important. Several other tick species were included in this study even though they are either not considered medically important or are not commonly found in Maryland. We felt it was critical to include these species to create a tool that would be useful to investigators in other regions of North America. Although the list of species used in this investigation is not exhaustive, we could speculate on the ability of the species-specific internal primers to anneal to template DNAs from species that were not tested, based on the alignments provided in Table 1.

Internal *Dermacentor*- and *Amblyomma*-specific primers were designed to amplify all species of these respective genera. One exception was *A. maculatum*. The expected *Amblyomma*-specific fragment could not be amplified from representatives of this species. The 16S gene alignment based on the *Amblyomma* sequences from GenBank illustrates that the internal primer region is highly polymorphic with multiple base changes in *A. maculatum* compared to *A. amblyomma*. Attempts at creating a primer with degenerate bases at the predicted mutation sites was not successful (data not shown). The distribution of *A. maculatum* is limited to the coastal regions of the southeastern United States. Specimens have been found at remote inland locations but populations do not generally become established. In addition, this tick is not common throughout much of the eastern United States.

Due to limited variability in the small 16S rRNA target sequence on which this diagnostic is based, a conserved primer set could not be created to differentiate between *Rhipicephalus*, *Haemaphysalis*, and *Ixodes*. This was partially overcome by using a restriction enzyme, *TauI*, which specifically cleaves *Rhipicephalus*, leaving both *Haemaphysalis* and *Ixodes* template DNAs uncut (Fig. 4). Further differentiation of *Ixodes* ticks should not be problematic as the Prostriata can be easily differentiated from the Metastriata using traditional morphological characteristics on all life stages, both engorged and un-engorged. Therefore, the derived PCR-RFLP-based diagnostic is only intended for differentiation of the four Metastriate genera; *Dermacentor*, *Amblyomma*, *Rhipicephalus*, and *Haemaphysalis* (Fig. 2). The molecular diagnostic described here will allow investigators to confidently discriminate between these Metastriate genera, quickly, easily and inexpensively, either as a confirmation of morphological identifications or verification for material processed in the laboratory for vector genetics or pathogen surveillance.

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Fig. 1. Schematic of the multiplex primer combination with predicted amplification product size.

Fig. 2.

PCR amplification products from the multiplex reaction. Lanes 1–8 are amplified products from *Dermacentor* species (lanes 1–3: *D. variabilis* adult, nymph and larvae; lanes 4–6: *D. albipictus* adult, nymph and larvae; lane 7: *D. andersoni* adult; lane 8: *D. occidentalis* adult). Lanes 9–13 represent amplification from *Amblyomma* species (lanes 9–11: *A. americanum* adult, nymph and larvae; lanes 12–13: *A. maculatum* adult and nymph). Lanes 14–16 are *Haemaphysalis leporispalustris* adult, nymph and larvae. Lanes 17–18 are *Rhipicephalus sanguineus* adult and nymph specimens. Lanes 19–21 are *Ixodes scapularis* adult, nymph, and larvae. The ladder is a 100-bp marker. L, ladder; NC, negative control.

TauI	G 5'-G C C G^C-3' 3'-C^G G C G-5' C
<i>Rhipicephalus</i>	
<i>R. appendiculatus</i>	G C G G C
<i>R. bursa</i>	G C G G C
<i>R. pusillus</i>	G C G G C
<i>R. sanguineus</i>	G C G G C
<i>R. turanicus</i>	G C G G C
<i>Haemaphysalis</i>	
<i>H. cretica</i>	G C G A T
<i>H. inermis</i>	G C G A T
<i>H. leporispalustris</i>	G C G A T
<i>H. punctata</i>	G C G A T
<i>Dermacentor</i>	
<i>D. andersoni</i>	G C A A C
<i>D. albipictus</i>	G C A A C
<i>D. hunteri</i>	G C A A C
<i>D. halli</i>	G C A A C
<i>D. imitans</i>	G T A A C
<i>D. marginatus</i>	G C A A C
<i>D. nitens</i>	G C A A C
<i>D. occidentalis</i>	G C A A C
<i>D. variabilis</i>	G C A A C
<i>Amblyomma</i>	
<i>A. americanum</i>	G C G A T
<i>A. cajennesse</i>	G C G A T
<i>A. glauerti</i>	G C G A T
<i>A. maculatum</i>	G C G A T
<i>A. ovale</i>	G C G A T
<i>A. tuberculatum</i>	G C G A T
<i>Ixodes</i>	
<i>I. scapularis</i>	G C G A T

Fig. 3.

Ribosomal 16S fragment alignment of the restriction enzyme digestion region (nucleotide 272–276) for TauI restriction enzyme of several species of hard ticks. Species names in bold were tested during this analysis.

Fig. 4. Restriction digestion of genera that were not differentiated with multiplex PCR primer set. Samples were digested with *TauI* restriction enzyme. D, digested; U, undigested.

Table 1
Tick Species, Genbank Accession Numbers Of Sequence Data Used To Design Diagnostic Primers, and Alignment Of Priming Region For Each Species

Species	Origin	Accession number	16sDvF primer (nt 334–360)	Reference
<i>Dermacentor</i>			AATGA–TTATTTGA–GAAAAATAC	
<i>D. albipictus</i>	CA, USA	AF001231	.....-.....-.....	Crosbie et al., 1998
<i>D. albipictus</i>	WA, USA	AF001232	. G...- C.....- A.....	Crosbie et al., 1998
<i>D. albipictus</i>	WA, USA	AF001233	.....- A.....- A.....	Crosbie et al., 1998
<i>D. albipictus</i>	VA, USA	AY676458	.....- A..... G. G.....	Unpublished ^a
<i>D. andersoni</i>	KS, USA	L34299	.....- A.....- TA.....	Black & Piesman, 1994
<i>D. halli</i>	TX, USA	AF001247	.....-..... A.....	Crosbie et al., 1998
<i>D. hunteri</i>	CA, USA	AF001246	.....-..... C.....- A.....	Crosbie et al., 1998
<i>D. imitans</i>	Venezuela	AF001248	.....- A.....- A.....	Crosbie et al., 1998
<i>D. marginatus</i>	unknown	Z97879	.....-..... A.....	Mangold et al., 1997
<i>D. nitens</i>	ID, USA	AF001249	. T...- A. C... A.....	Crosbie et al., 1998
<i>D. occidentalis</i>	CA, USA	AF001252	.....- A..... A.....	Crosbie et al., 1998
<i>D. variabilis</i>	KS, USA	L34300	.....-.....-.....	Black & Piesman, 1994
<i>D. variabilis</i>	CA, USA	AY010235	.....- A.....- A.....	de la Fuente et al., 2001
<i>D. variabilis</i>	FL, USA	AY010237	.....-.....-.....	de la Fuente et al., 2001
<i>D. variabilis</i>	VA, USA	AY010241	.....- C.....-.....	de la Fuente et al., 2001
<i>D. variabilis</i>	ID, USA	AY010238	.....- A..... G.. A.....	de la Fuente et al., 2001
<i>Haemaphysalis</i>				
<i>H. cretica</i>	Iseral	L34308	.....- T. A...- A.....	Black & Piesman, 1994
<i>H. inermis</i>	Slovak Rep	U95872	.....-..... A... TT-.....	Norris, et al., 1999
<i>H. leporispalustris</i>	Georgia	L34309	.....-.....- T.....	Black & Piesman, 1994
<i>H. punctata</i>	unknown	Z97880	.....- A.. AA...- T.....	Mangold et al., 1997
<i>Rhipicephalus</i>				
<i>R. appendiculatus</i>	UK	L34301	.....- AA...-.....	Black & Piesman, 1994
<i>R. bursa</i>	unknown	AJ002956	.....-..... A...-.....	Marquez et al., 1997
<i>R. pusillus</i>	unknown	AJ002957	.....- CA.. T--- T.....	Marquez et al., 1997
<i>R. sanguineus</i>	Iseral	L34302	.....-..... A.. T--- T.....	Black & Piesman, 1994
<i>R. sanguineus</i>	unknown	Z97884	.....-..... A.. T--- T.....	Mangold et al., 1997
<i>R. turanicus</i>	Isreal	L34303	.....-..... A.. T--- T.....	Black & Piesman, 1994
<i>Amblyomma</i>			.. C. G- AC. C... G.....	
<i>A. americanum</i>	KS, USA	L34313	.. C. G- AC. C... G.....	Black & Piesman, 1994
<i>A. americanum</i>	TX, USA	L34314	. GC. G- A. A... G- T.....	Black & Piesman, 1994
<i>A. cajennense</i>	OK, USA	L34317	.... G- A. AA...-.....	Black & Piesman, 1994
<i>A. glauerti</i>	Australia	U95853	. T.. G..... A.....	Norris et al., 1999
<i>A. maculatum</i>	OK, USA	AY676459	. T.. G.....	Unpublished ^a

Species	Origin	Accession number	16sDvF primer (nt 334–360)	Reference
<i>A. macuatum</i>	OK, USA	L34318	. T.. G-... GA...- T.....	Black & Piesman, 1994
<i>A. ovale</i>	Paraguay	AF541255	. T.. G-... AA... AT.....	Guglielmone et al., 2003
<i>A. tuberculatum</i>	GA, USA	U95856		Norris, et al., 1999
<i>Ixodes</i>			. GA...-... AA...-.....	
<i>Ixodes scapularis</i>	NC, USA	L43863	.....-.....-...-.....	Black & Piesman, 1994
<i>Amblyomma</i>			16sAmR Primer (nt 339–360) CGG-ACACTTGG-AAAAAATAC	
<i>A. americanum</i>	Brazil	AF541254	G..- TA. AA.. A-TC.....	Guglielmone et al., 2003
<i>A. americanum</i>	KS, USA	L34313	...-.....-.....	Black & Piesman, 1994
<i>A. americanum</i>	TX, USA	L34314	...-.....-.....	Black & Piesman, 1994
<i>A. cajennense</i>	OK, USA	L34317	...- TA. A...- T.....	Black & Piesman, 1994
<i>A. glauerti</i>	Australia	U95853	T..- TA. AA.. A-.....	Norris, et al., 1999
<i>A. maculatum</i>	OK, USA	AY676459	T..- TT. T... A--G.....	Unpublished ^a
<i>A. macuatum</i>	OK, USA	L34318	T..- TT . T... A--.....	Black & Piesman, 1994
<i>A. ovale</i>	Paraguay	AF541255	T..- TT. GA.. A . AT.....	Guglielmone et al., 2003
<i>A. tuberculatum</i>	GA, USA	U95856	T..- TT. AA.. AA . T.....	Norris, et al., 1999

Species in **bold** were tested in this analysis. nt, nucleotide position based on the full-length 16S rRNA fragment.

^aNewly sequenced tick isolate used to optimize the PCR primers for the multiplex PCR analysis.

Table 2
Primers Used In The Multiplexed PCR

Primer	Sequence 5'→3'	Genus
16S+1	CTGCTCAATGATTTTTTAAATTGCTGTGG	All
16S-1	CCGGTCTGAACT AGATCAAGT ^a	All
	LR-J-12887 (Simon et al. 1994) with an A substitution at position 20	
16SAmr	GTATTTTTTCCAAGTGTCCG	<i>Amblyomma</i>
16SDvF2	AATGATWATTTGRGAAAATAC	<i>Dermacentor</i>

^aLRJ, etc.