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MOLECULAR PHYLOGENETIC RELATIONSHIPS OF NORTH AMERICAN *DERMACENTOR* TICKS USING MITOCHONDRIAL GENE SEQUENCES

by

KAYLA PERRY

(Under the Direction of Quentin Fang and Dmitry Apanaskevich)

ABSTRACT

Dermacentor is a recently evolved genus of hard ticks (Family Ixodiae) that includes 36 known species worldwide. Despite the importance of *Dermacentor* species as vectors of human and animal disease, the systematics of the genus remain largely unresolved. This study focuses on phylogenetic relationships of the eight North American Nearctic *Dermacentor* species: *D. albipictus, D. variabilis, D. occidentalis, D. halli, D. parumapertus, D. hunteri,* and *D. andersoni*, and the recently re-established species *D. kamshadalus*, as well as two of the Neotropical *Dermacentor* species *D. nitens* and *D. dissimilis* (both formerly *Anocentor*). We sequenced portions of the mitochondrial cytochrome oxidase subunit I (COI) gene, and the ribosomal 12S and 16S genes from the largest sampling of North American *Dermacentor* ticks analyzed to date. In all analyses, we found that North American *Dermacentor* ticks form a monophyletic lineage, and that all four species of one-host *Dermacentor* ticks also form a monophyletic lineage within the genus. The placement of the former *Anocentor* species, *D. nitens* and *D. dissimilis* in Dermacentor is also well supported. The winter tick, *Dermacentor albipictus*, has a complex structure in all analyses that warrants further study into the possibility of a species complex. *Dermacentor kamshadalus*, formerly a synonym of *D. albipictus*, shows the same complex structure under analysis of these three mitochondrial genes, and should also be further molecularly examined.

MOLECULAR PHYLOGENETIC ANALYSIS OF NORTH AMERICAN *DERMACENTOR* TICKS USING MITOCHONDRIAL GENE SEQUENCES

by

KAYLA PERRY

B.S., Georgia Southern University, Statesboro, GA 2011

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

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MOLECULAR PHYLOGENETIC ANALYSIS OF NORTH AMERICAN DERMACENTOR TICKS USING MITOCHONDRIAL GENE SEQUENCES

by

KAYLA PERRY

Major Professors: Quentin Fang

Dmitry Apanaskevich

Committee: Lance Durden

Electronic Version Approved:

May 2014

DEDICATION

This body of work is lovingly dedicated to my parents, who have always supported and believed in me, and who taught me through their example to seek knowledge for its own sake, to work hard, to play hard, and to "not sweat the small stuff."

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I would like to thank my advisors, Dr. Quentin Fang and Dr. Dmitry Apanaskevich, for teaching me about ticks, about science, and about life. I would like to thank my lab mate, Ashlin Reid, for being my research collaborator, late-night lab friend, and the best conference travel companion I could have asked for. I would like to thank my committee member Dr. Lance Durden for his kind patience and for helpful edits and suggestions. I would like to thank all of those who contributed tick specimens for this project, including the United States National Tick Collection, Bob Henry and Randy Babbs of the Arizona Fish and Wildlife Department, Dr. Joel Hutcheson of the USDA, Dave Epstein, and Nick Macias. I would also like to thank Doug Walker for teaching me a plethora of computer skills that made constructing the figures for this thesis possible.

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Introduction

Ticks

Ticks are obligate, hematophageous ectoparasites, recognized as important veterinary and medical threats second in importance only to mosquitoes (Spach *et al.* 1993, Allan 2001, Parola and Raoult 2001). They have been found feeding on a wide variety of organisms including amphibians, reptiles, birds, and mammals (Bishopp and Trembley 1945, Oliver 1989, Burridge 2001, Smith *et al.* 2008). Ticks are in the kingdom Animalia, the phylum Arthropoda, the class Arachnida, and form the sub-class Acari with mites, and the order Ixodida. There are currently three recognized families of ticks: the Ixodidae (hard ticks, 702 species), the Argasidae (soft ticks, 193 species), and the Nuttalliellidae (monotypic, *Nuttalliella namaqua* in South Africa) (Keirans *et al*. 1976, Barker and Murrell 2004, Guglielmone *et al.* 2010, Mans *et al.* 2011). Ticks are presumed to represent early lineages of terrestrial arachnids and to have originated between the late Silurian and the late Cretaceous (443 – 65 million years ago) (Hoogstraal and Aeschlimann 1982, Lindquist 1984, Oliver 1989, Balashov 1994, Klompen *et al.* 1996, de la Fuente 2003). Blood-feeding behavior in ticks is believed to have evolved in an ancestral tick lineage, with the different mechanisms for hematophagy evolving through multiple independent events between 92 – 120 million years ago (Mans *et al.* 2002, Mans and Neitz 2004, Mans *et al.* 2011). With midguts that are uniquely suitable for pathogen survival and long feeding periods interspersed with periods of ingestion and regurgitation, ticks are well adapted for effective pathogen transmission (Parola and Raoult 2001), and can act as reservoirs of tick-borne diseases by maintaining pathogens in a population via transstadial (between life stages) and transovarial (from female to offspring) transmission (Parola and Raoult 2001).

Ticks are distributed worldwide but their greatest diversity is in the warmer regions. Ixodid ticks are among the most important vectors of disease-causing microorganisms to humans, and domestic and wild animals (Sonenshine, 1993). The genus *Dermacentor* Koch includes 35 known species distributed throughout the world, with 8 species endemic to North America, and 4 species endemic to Central America. Based on the U.S. National Tick Collection Database (USNTC), 25 species of *Dermacentor* have been shown to bite humans. *Dermacentor* tick species present in North America are important vectors of the causative agents of Rocky Mountain Spotted Fever (*Rickettsia rickettsii*), tularemia or "rabbit fever" (*Francisella tularensis*), Q Fever (*Coxiella burnetii*), Boutenneuse fever (*Rickettsia conori*), and the viruses that cause Colorado Tick Fever and Powassan encephalitis in humans, as well as Babesioses (*Babeisa caballi*, *B. canis*) in domesticated animals. Heavy losses sometimes occur in tick-infested domestic animals and -larger game animals, and infestations with *D. andersoni* or *D. albipictus* sometimes cause serious exsanguination anemia (Cooley, 1938). Females of some North American *Dermacentor* species, especially *D. andersoni* and *D. variabilis*, can also cause tick paralysis in humans and animals.

All ticks pass through four distinct life stages: 1) egg, 2) six-legged larva stage (sometimes referred to as "seed ticks"), 3) eight-legged nymph stage, and 4) the adult stage, in which the tick still has 8 legs. Although ticks are able to survive long periods of fasting, development from one life stage to the next and oviposition takes place only following attachment to a host and engorgement from a blood meal. Therefore, ixodid ticks engorge twice before arriving at the adult stage, and then engorge once as adults, and females die following oviposition (Bishopp and Trembley 1945). Thirty-one of the recognized *Dermacentor* species have a three-host life cycle in which a larva feeds on a host, typically a small mammal, and then

drops off until it has molted to the nymph stage where it must seek another host to parasitize before dropping off and molting into adulthood. Once the tick is an adult, it must find and feed on a third host before it can reproduce. Of the 12 *Dermacentor* species represented in the New World, however, 4 are known to utilize a one-host life cycle, in which only the larvae seek a host, and then remain attached to that host throughout both molts and mating, and females drop off to lay eggs (Yunker et al. 1986).

This study focuses on phylogenetic relationships of the eight North American Nearctic *Dermacentor* species: *D. albipictus, D. variabilis, D. occidentalis, D. halli, D. parumapertus, D. hunteri, D. andersoni*, and the recently re-established species *D. kamshadalus*, as well as two of the Neotropical *Dermacentor* species *D. nitens* and *D. dissimilis*.

Overview of North American *Dermacentor* species and previous studies

The three-host North American *Dermacentor* include the following species:

***1) Dermacentor variabilis* (Say, [1821]) (American dog tick)** is one of the most commonly encountered of the North American *Dermacentor* species, and has a wide, but disjunct distribution in the U.S., occurring from the Great Plain regions to the east coast and throughout California and southwestern Oregon, but is absent in the Rocky Mountain region. In Canada, *D. variabilis* is found in southeastern Saskatchewan and as far east as Nova Scotia. *D. variabilis* is has also been reported in northern Mexico. Larvae and nymphs feed predominantly on mice, particularly meadow mice and white-footed mice. Canids, including domestic dogs, are the principal hosts of adult *D. variabilis,* though a wide range of mammals including cattle, equids, deer, opossums, and rabbits can also be parasitized by this species (Burgdorfer, 1969). In the only molecular phylogenetic work completed to date on *D. variabilis*, Crosbie et al. (1998)

reported strong bootstrap support for the monophyly of this species. However, only three sequences of 16S were used, one from a tick collected in California, one from a colony maintained by Rocky Mountain Laboratories, and one sequence downloaded from GenBank that was part of a 1994 study by Black and Piesman that does not specify the geographic region where the tick was collected. Due to the separation between the two ranges of *D. variabilis* (one in the eastern U.S. and the other far western), it is impossible to confidently judge the amount of variation within this species without sampling from both regions.

2) Dermacentor occidentalis **Marx, 1892 (Pacific Coast tick)** is a common tick in wooded areas within its relatively restricted range in the states of California and Oregon, and in limited Western regions of Canada and Mexico. In Oregon, it is found west of the cascade mountains and as far north as Yachats. In California, it is found in most of the wooded areas of the state west of the Sierra Nevada Mountains, but is reportedly absent from northeastern California (Kohls 1970). The principal hosts of adult *D. occidentalis* are black-tailed deer (*Odocoileus hemionus columbianus, O. hemionus sitkensis*), but it can also parasitize a wide range of mammalian hosts including cattle, equids, humans, dogs, and rabbits (Kohls 1970). The immatures of *D. occidentalis* feed most frequently on ground squirrels (*Spermophilus beecheyi, S. douglasii*), but have also been collected from a variety of small mammals including chipmunks and wood rats (Kohls 1970). Crosbie et al. (1998) analyzed three specimens of *D. occidentalis* and found high levels of support for monophyly within this species. No other molecular phylogenetic analysis has been published on *D. occidentalis* thus far.

3) ***Dermacentor parumapertus*** **Neumann, 1901 (Rabbit dermacentor)** occurs in arid areas and is found in association with rabbits even under extreme desert conditions, in every month of the year (Burgdorfer 1969). *D. parumapertus* has been collected in 11 states in the

Southwestern U.S. (Cooley 1938), but is most abundant in Texas and southern New Mexico, Arizona, California, Nevada, and Utah (Burgdorfer 1969, James et al. 2006). The adults feed almost exclusively on jack rabbits (*Lepus californicus*) and cottontail rabbits (*Sylvilagus* spp.), and these serve as the principal hosts for immature stages as well, although larvae and nymphs will also feed on available species of rodent, particularly kangaroo rats (*Dipodomys ordii, Dipodomys microps*) (Burgdorfer, 1969).

4) ***Dermacentor andersoni*** **Stiles, 1908 (Rocky Mountain wood tick)** is distributed in the mountainous regions of the Western U.S. and in the southern parts of British Columbia, Alberta and Saskatchewan in Canada (Burgdorfer 1969, James et al. 2008). *D. andersoni* is not host-specific, and usually feeds on a variety of rodents and other small mammals during its nymph and larval stages, while adults typically parasitize larger mammals such as cattle, horses, dogs, deer, bears, and humans (Burgdorfer 1969).

In the only molecular phylogenetic work done on *Dermacentor andersoni* and *Dermacentor parumapertus* thus far, three specimens of each species grouped together to form a single clade with 99% bootstrap support and no resolved topology within the clade under maximum parsimony, maximum likelihood, or neighbor-joining phylogeny (Crosbie 1998). However, as mentioned, that study analyzed only a short sequence of 16S, and these two species may require larger sampling and analysis of more gene regions to find enough separation to allow for their eventual molecular identification.

5) ***Dermacentor hunteri*** **Bishopp, 1912 (Bighorn Sheep Tick)** adults parasitize desert bighorn sheep (*Ovis canadensis* Shaw) almost exclusively, and often at high prevalence and density. The range of this species is as restricted and fragmented as that of its ungulate host (Crosbie et al. 1997). Desert bighorn sheep inhabit isolated mountain ranges in the southwestern

U.S. (Monson 1980) and *D. hunteri* are carried between populations in different mountain ranges by highly vagile rams (Bleich et al. 1990). Immature *D. hunteri* feed primarily on desert wood rats (*Neotoma lepida*), so the range of this tick is further limited to areas where suitable hosts are simultaneously available for all stages of its development (Crosbie et al. 1997). This species was the primary focus of the most comprehensive molecular phylogenetic analysis of North American *Dermacentor* to date. Crosbie et al. (1998) tested 11 specimens of *D. hunteri* from various regions of their range finding, as they had expected, high levels of support for the monophyly of this species and little genetic variation among individuals or populations. There was some discrepancy, however, in the correct position of this monophyletic species within the genus, as it grouped with *D. albipictus* and *D. nitens* on the maximum parsimony and maximum likelihood trees, but grouped with a clade formed by *D. andersoni* and *D. parumapertus* on the neighbor-joining tree (Crosbie et al. 1998). Although the sampling of *D. hunteri* in the previous study was comprehensive, it is possible that this species' position within the genus can be better elucidated using a longer fragment of 16S, incorporating data from additional genes, and analyzing a larger sample of specimens from other *Dermacentor* species.

6) ***Dermacentor halli*** **McIntosh, 1931(Peccary tick)** adults feed predominantly on collared peccaries (*Tayassu tajacu*), and although this species has only formally been reported from southern Texas (Cooley 1938), it is likely that *D. halli* can be found anywhere in the expanding range of the collared peccary, which includes parts of New Mexico and Arizona and northern Mexico. The phylogenetic position of *Dermacentor halli* has only been molecularly examined once, and using just one specimen that formed a clade with three specimens of *D. variabilis* under maximum parsimony and maximum likelihood analyses, but formed an independent clade under neighbor-joining analysis. Bootstrap analysis was unable to resolve

these differences, leaving the proper phylogenetic position of *D. halli* within *Dermacentor* still unresolved (Crosbie 1998).

The one-host *Dermacentor* ticks include the following species:

1) ***Dermacentor albipictus*** **(Packard, 1869) (winter tick)** has the broadest geographic range of any New World *Dermacentor*, stretching from southern Canada to Mexico and Central America, and disjointly covering most of the contiguous U.S. The winter tick feeds mostly on large ungulates, including moose, deer, and bighorn sheep, and often occur in large numbers on the host. The taxonomy of *D. albipictus* has been debated since Packard first formally described two forms of the winter tick as *Ixodes albipictus* and *Ixodes nigrolineatus* (Packard 1869). The winter tick was later placed in the genus *Dermacentor* by Banks in 1907. Cooley (1938) did not consider the morphological difference between these two forms to be significant enough to warrant recognition as two species, with the difference being viewed as a result of more transparent cuticle in *Dermacentor nigrolineatus.* Cooley's (1938) synonymy of *D. nigrolineatus* under *D. albipictus* was supported by their ecological similarity as one-host ticks that share the same host ranges and are active at the same time of year. Ernst and Gladney (1975) later showed that the two forms of *D. albipictus* could hybridize and produce viable offspring. Nevertheless, some authors continued to recognize *D. nigrolineatus* as a distinct species (Bishopp and Trembley 1945, Camicas et al. 1998). Using mitochondrial 16S rDNA gene sequences, Crosbie et al. (1998) discovered significant genetic variation among *D. albipictus* individuals that suggests the presence of a species complex, with one specimen grouping more closely to another one-host tick, the tropical horse tick, *D. nitens,* than to other *D. albipictus* specimens. The only other published molecular phylogenetic work done on this question also revealed deep mitochondrial DNA lineage divergences within this species, but concluded that these

divergences are not enough to indicate distinct species (Leo et al. 2010). However, Crosbie's (1998) study only included sequences from four specimens of *D. albipictus*, one from New Mexico, one from California, and two from Washington State. Leo et al.'s (2010) study included specimens exclusively from in and around Alberta. *Dermacentor nigrolineatus* was originally described in New York State, and the morphological characteristics included in that original description (Packard 1896) are more commonly observed in populations in the eastern U.S. Therefore, the limited sampling of previous studies may have failed to detect the overall intra-specific genetic diversity in *D. albipictus,* and may restrict the ability to delimit its species boundaries. The extensive but fragmented distribution and broad host specificity seen in *D. albipictus* may result in the formation of isolated populations with disrupted gene flow, leading to population differentiation and eventual speciation. A very large and representative sampling of the winter tick's full range is necessary to investigate the possibility of a species complex.

2) ***Dermacentor kamshadalus* Neumann, 1908** was also formerly included within the species *Dermacentor albipictus*, but was recently morphologically reinstated as a valid species (Apanaskevich, 2013). *D. kamshadalus* occurs in the northwestern U.S., particularly in the mountain ranges of Idaho and Montana and primarily parasitizes rocky mountain goats (Apanaskevich 2013). Due to the recentness of its re-establishment, no published molecular phylogenetic work has been performed to support its taxonomic reinstatement or to determine its relationship to other one-host ticks within the genus.

Neotropical *Dermacentor* examined:

Although this study focuses primarily on the Nearctic New World *Dermacentor* species, two Neotropical species, *Dermacentor nitens* and *Dermacentor dissimilis*, were also included

because they are the only other species within *Dermacentor* to have a one-host life cycle, making their inclusion imperative to answering the question of whether this adaptation arose only once within the genus.

***Dermacentor dissimilis* Cooley, 1947** occurs mostly in southern Mexico and Guatemala and parasitizes mostly equids (Cooley 1947). Very little work has been done on this species, although Borges et al. (1998) found it to be most closely related to *Dermacentor albipictus* via analysis with morphological numerical taxonomy. The present study is the first to molecularly investigate the taxonomic position of *D. dissimilis*.

***Dermacentor nitens* Neumann, 1897 (Tropical Horse Tick)** is distributed from the southern U.S. to northern Argentina. *D. nitens* predominantly parasitizes equids, but may also feed on cattle, sheep, goat, deer, and hogs (Yunker et al. 1986). The molecular 16S study conducted by Crosbie et al. (1998) included one specimen of *D. nitens* that grouped closely with specimens of *D. albipictus* in all three non-bootstrapped analyses. This grouping led Crosbie et al. to suggest the possibility that 1-host specificity evolved a single time in *Dermacentor*. The present study is the first to molecularly examine all four of the recognized one-host *Dermacentor* species for the possibility of monophyly, as Crosbie et al. (1998) did not include specimens of *D. dissimilis* or *D. kamshadalus*.

Additionally, *Dermacentor nitens* was placed in the genus *Otocentor* by Cooley in 1938, and both *D. nitens* and *D. dissimils* formerly comprised the separate genus *Anocentor* Schulze, which was later designated as a subgenus to *Dermacentor* (Diamant and Strickland 1965). Borges et al. (1998) asserted that *D. dissimilis* could be legitimately included within *Dermacentor*, and that *D. nitens* still formed the monotypic genus *Anocentor*, finding through numerical taxonomy that *D. nitens* was more closely related to the genus *Rhipicephalus* than to

Dermacentor. Broad family level molecular work on ticks led Barker and Murrell (2002) to conclude that *D. nitens* should be considered true *Dermacentor*. No specific molecular work has yet been published to test whether the inclusion of *D. dissimilis* and *D. nitens* in the genus *Dermacentor* is supported.

The only molecular phylogenetics study previously completed involving most of these species was done by Crosbie et al. (1998). They sequenced a 300 base pair region of the mitochondrial 16S gene for 30 New World *Dermacentor* specimens. Therefore, relationships between *Dermacentor* species and clearly defined species and genera limits have yet to be adequately resolved. The aim of this work is to use broad, all-inclusive sampling of each species across the entirety of their respective ranges, as well as multiple gene loci, to complete the most comprehensive molecular study of New World *Dermacentor* to date.

Molecular Phylogenetics and Species Identification

Correctly determining phylogenetic relationships and clearly delimiting species is important in the study of ticks as closely related tick species and even different populations within a tick species can differ in their ability to transmit pathogens (Anderson 2002, Baker 1998). Historically, theories about tick evolution and systematics have been based on morphology, host associations, and life history. However, due to high levels of intra-specific variation and inter-specific overlap of many morphological traits and hosts exploited, strictly morphological delimitation among ticks can be difficult and unreliable. Analysis of parasite biology including geographical distribution, host, behavior, varying pheromone-induced responses, and symbiont presence may provide alternate methods to vector species delimitation (Lumley and Sperling 2011). However, these alternatives are applicable only if the traits can be

definitively associated with just one species, and several authors have concluded that hybridization experiments or molecular markers may be necessary to fully delimit and distinguish such tick species (Zahler and Gothe 1997, Zahler et al. 1995, Baker 1998, Fukunaga et al. 2000, Dergousoff and Chilton 2007). Additionally, information on parasite genetic diversity and evolutionary history can potentially serve as a tool for accurate identification of species and for increasing our understanding of host-parasite-pathogen interactions (Stockwell and Leberg 2002, Armstrong and Ball 2005). Knowledge of genetic diversity and complete species delimitation is a prerequisite for molecular identification techniques such as DNA barcoding, which is a system designed to provide rapid, accurate, and automatable identifications by using short standardized gene regions as internal species tags (Hebert et al. 2003). Implementation of effective targeted vector control requires this kind of quick and reliable identification of vector species (Rosen 1986, Ball and Armstrong 2008) that is not always possible based solely on morphology, due to the presence of cryptic species (Bickford *et al.* 2007) and the fact that morphological identifications are intrinsically qualitative and dependent on the investigator's familiarity with the organism, specimen quality, and the life stage being identified (Hebert *et al.* 2003).

Fortunately, advances in sequencing techniques have presented us with an efficient method for species delimitation and identification that can potentially be quantitatively standardized. Although this method has some limitations and must be examined further for reliability, it can be very useful for pest species identification (Armstrong and Ball 2005, Rubinoff *et al.* 2006). Molecular identification techniques are especially useful when dealing with specimens of poor quality or juvenile stages (Hebert *et al.* 2003), and can allow us to overcome problems with specimen quality and size, and may potentially differentiate cryptic

species, which is particularly important for identifying closely related and morphologically similar organisms that exhibit varying efficiency in causing diseases or transmitting pathogens (Maingon *et al.* 2008, Estrada-Peña *et al.* 2009). Extensive sampling across a species' geographical range and the use of multiple genes allows us to account for as much intra-specific genetic diversity as possible (Elias *et al.* 2007). Such genetic information can be applied in combination with other identification methods to delimit and identify pest species via an integrative approach (Wiens 2007, Schlick-Steiner *et al.* 2010).

In this study, we chose to sequence portions of the mitochondrial 16S, 12S, and COI genes from the most comprehensive collection of North American *Dermacentor* ticks molecularly analyzed to date. Each species is represented by multiple specimens from all parts of its distribution.

Mitochondrial 12S, 16S, and COI genes

Mitochondrial DNA has been widely used in animal phylogenetic analysis. The animal mitochondrial genome is small and usually contains genes for 13 proteins, 22 tRNAs, 2 rRNAs, and 1 or 2 control regions for a total of 36 to 37 genes (Hwang and Kim 1999). Typically, the mitochondrial genome also contains at least one sequence of variable length that does not encode for any gene, but is a control region that, in vertebrates and insects, is known to include elements that regulate and initiate mtDNA replication and transcription (Hwang et al. 1998). Mitochondrial genes occur in large numbers in each cell, but usually all of these copies have the same sequence due to the population bottleneck created by exclusive maternal inheritance (Simon et al. 1994). Mitochondrial DNA evolves much faster than the nuclear genome, and as a result, most of the mitochondrial protein coding regions have been used to examine phylogenetic

relationships in the lower levels such as families, genera, species, or populations. The evolutionary rate of parasites, and thus the degree of sequence variation, of selected molecular markers or gene regions is considerably faster than that of independent organisms (Hwang et al., 1998). Therefore, to elucidate phylogenetic relationships among parasites such as ticks, more conserved (slowly evolving) gene or gene regions should be used in addition to those generally used for independent organisms (Hwang and Kim, 1999). Mitochondrial genes fall into two categories; ribosomal genes and protein-coding genes. The large subunit 16S and small subunit 12S RNA genes are the only two mitochondrial ribosomal genes that are not separated by internal transcribed spacers (Cruickshank 2002).

Mitochondrial COI and 16S rDNA genes are the most commonly used molecular markers to infer species level phylogenetic relationships in other taxa, such as brachyuran crabs (Harrison 2004). The 16S gene has been shown to be more phylogenetically informative than COI for determining relationships between species (Harrison 2004), and to be more variable and phylogenetically informative that the mitochondrial 12S gene in both interspecific and intraspecific studies. The mitochondrial 16S RNA gene has been used repeatedly to test phylogenetic hypotheses in other arthropod taxa, such as black flies (Xiong and Kocher, 1993), leafhoppers (Fang et al., 1993), mites (Johanowicz and Hoy 1996), ixodid ticks (Black and Piesman (1994), North American *Dermacentor* tick species (Crosbie et al. 1998, Leo et al. 2010), and tick species in the *Ixodes ricinus* complex (Xu et al. 2003). Published studies suggested that mitochondrial 16S RNA genes are suitable for resolving phylogenetic relationships in ticks below the subfamilial level.

The 12S rDNA gene has been used in multiple studies of acarine phylogeny (Black and Piesman 1994, Beati and Kierans 2001), and 12S ribosomal DNA tick phylogenies have been

shown to resolve relatively recent speciation events better than earlier ones (Murrell et al. 1999, Norris et al. 1999).

However, for ribosomal genes, like 16S and 12S, which are not translated into proteins, and thus do not have the three base-pair codon structure, sequence alignment can be much more difficult, and uncertainty in the alignment can lead to uncertainty in the phylogeny (Cruickshank 2002, Brower and DeSalle 1994).

The mitochondrial cytochrome oxidase subunit I gene (COI) is also widely used for phylogenetic analysis in a variety of organisms. Due to the high rate of substitution occurring in the third codon positions (wobble positions) of protein coding genes, the DNA sequences of protein coding genes, including COI, have frequently been used for species level or population level phylogeny (Navajas et al., 1996). Anderson and Trueman (2000) used COI to show that *Varroa jacobsoni* is a complex of two morphologically indistinguishable species infesting the same host, *Apis cerana*, but with only one of those species being able to transfer to another host. A good example of the use of mtDNA COI sequences for phylogenetic analysis of species delineation was carried out in the family Tetranychidae of Spider mites (Ros and Breeuwer 2007). Additionally, an eventual goal for this work, once each species boundary has been fully delimited and phylogenetic relationships are established, is to create DNA barcodes to be used for molecular identification for all species of *Dermacentor*, and COI has been proposed as the most appropriate gene region for DNA barcoding in animals (Hebert et al. 2003, Armstrong and Ball 2005, Stoeckle 2003).

Many of the ticks included in this study were museum specimens subjected to long term storage in ethanol, making use of nuclear genes with any consistency very difficult. The mitochondrial genome was chosen in hopes of having usable molecular data from a larger and

more diverse set of samples than has ever been done before in this genus, and the genes were chosen with the intention of simultaneously ascertaining interspecific relationships within *Dermacentor* and evaluating the intraspecific variation and possible groupings within and among populations of each species.

Significance and Objectives

Despite the medical and veterinary importance of *Dermacentor* species, their systematics and phylogenetics are poorly resolved. To date only a few of the several species of *Dermacentor* have been included in phylogenetic analyses, and the end-purpose of those analyses was not the reconstruction of the phylogeny of *Dermacentor* (Klompen et al. 1996, 1997, 2000, Barker and Murrell 2002). Molecular data are generally sparse and available only for a few of the more common species. A compilation of all of these still does not permit us to understand the relationships within the genus. As important medical and veterinary pests, it is critical to be able to identify tick species accurately and increase understanding of interactions between ticks and their environment. Such knowledge will be invaluable for implementing efficient monitoring and control programs. Information on parasite genetic diversity can potentially serve as a tool for accurate identification of pest species and for increasing the understanding of host-parasite-pathogen interactions (Stockwell and Leberg 2002, Armstrong and Ball 2005, Magalhães *et al.* 2007).

Objectives of Research:

1) Determine whether North American *Dermacentor* ticks form a monophyletic lineage.

2) Determine whether all *Dermacentor* ticks that employ a one-host life cycle form a monophyletic lineage.

3) Determine whether the inclusion of former *Anocentor nitens* and *Anocentor dissimilis* in the genus *Dermacentor* is molecularly supported.

4) Determine whether variation within *Dermacentor albipictus* indicate that it is actually a complex of closely related species.

5) Determine whether molecular support exists for the recent morphological reinstatement of *Dermacentor kamshadalus* as an independent species.

Materials and Methods

Tick Collection and Sampling

A total of 86 individual North American *Dermacentor* ticks were analyzed in this study. Thirty specimens were obtained from the U.S. National Tick Collection housed at Georgia Southern University. Others were requested from and donated by government workers, friends, and acquaintances across the country. All (9) specimens of *Dermacentor hunteri* used in this study were collected by Bob Henry and Randy Babbs of the Arizona Fish and Wildlife Department. Dr. Joel Hutcheson of USDA contributed multiple specimens of *D. albipictus, D. halli,* and *D. nitens.* Two of the *D. parumapertus* specimens were collected from road killed Jackrabbits during a family vacation. Each species was sampled from multiple geographic locations representing their entire range. Table 2 lists the individual ticks analyzed and their respective sources and collection localities.

Sixty-nine tick specimens, including all of those obtained from the U.S. National Tick Collection, had been stored in 70%-100% ethanol. When asking people to collect ticks for this study, they were provided with collection vials containing RNAlater RNA stabilization Reagent (Qiagen), and instructed to place live ticks into the solution and ship at room temperature. The 12 ticks preserved in this manner provided higher quality DNA extractions. Three specimens were placed directly in a -20ºC freezer alive, yielding even better quality DNA, and 2 specimens represent DNA extracted directly from live ticks, which yielded the highest quality nucleic acids

extractions of all. The most challenging specimens to extract quality DNA from were museum specimens that had been stored in ethanol for long periods of time, sometimes up to 90 years.

Gradient Relaxation of Alcohol Preserved Ticks

It was determined early on that special care would have to be taken when extracting nucleic acids from tick specimens that had previously been subjected to long term storage in ethanol. Ethanol stored ticks were hard and had brittle tissues, and residual ethanol in tissues can inhibit polymerase during PCR, so a gradient relaxation technique was implemented for these specimens. The hard cuticles of individual ticks were sliced open with a sterile scalpel blade under a dissecting microscope. Attempts were made to remove as much digested blood as possible from engorged females. Sliced ticks were then placed in 1.5mL microcentrifuge tubes containing 80% ethanol solution and 2 µL of Proteinase K and placed on a shaker for 30 minutes before being moved to tubes containing 60% ethanol, 40%, 20%, and 0% for 30 minutes each and always with the addition of 2 µL of Proteinase K.

DNA Extraction

DNA was easily obtained from all frozen specimens and all freshly collected specimens stored in RNAlater solution, whereas the yield of DNA from alcohol preserved ticks, especially those subjected to many years of ethanol storage, was highly variable. Specimen quality varied significantly even among samples of similar age, which may have been influenced by collection method and handling before preservation.

Total DNA was extracted from individual tick specimens using Epicentre Master Complete DNA & RNA Purification Kits (Epicentre Technologies, Madison, Wisconsin), according to manufacturer's protocols. Specimens placed in 1.5mL microcentrifuge tubes with

350 µL of 2X T&C (Tissue and Cell) Lysis Solution and 3 µL of Proteinase K. Ticks were then homogenized in this solution using either an electric homogenizer or plastic pestles. Samples were incubated at 55°C while being periodically subjected to grinding with pestles, from 6 to 24 hours, as needed to completely homogenize the sample. The temperature on the heating block was raised to 85°C for the final 15 minutes of incubation, in order to facilitate more protein denaturation. Samples were then placed on ice for 5 minutes. 150 µL of MasterPure-complete (MPC) Protein Precipitation Reagent (Qiagen) was then added to each sample before they were subjected to 10 minutes of centrifugation at 14,000 rpm at 4°C. If the resulting pellet was loose, clear, or small, an additional 25 µL of MPC was added and the sample was centrifuged for 10 more minutes under the same conditions. The supernatant was then transferred to a second 1.5 mL microcentrifuge tube and the pellet was discarded. 500 µL of 100% isopropanol was added to the recovered supernatant, and the tube was inverted 30-40 times before being centrifuged for 10 minutes at 14,000 rpm at 4°C. The resulting supernatant was then poured or pipetted off, with care not to disturb the pellet. 1mL of 75% ethanol was then added to the tube for rinsing and each sample was centrifuged for 2 minutes at 14,000 rpm at 4°C. The ethanol was then poured or pipetted off with care not to disturb the pellet, and then the pellet as dried in a 37°C incubator with the lid open for 10-20 minutes, or until all of the ethanol had evaporated. The pellet was then resuspended in 40 µL of ddH_20, and the extraction was visualized on a 1% agarose gel (5 µL of sample + 5 µL of loading buffer). Extracted DNA was stored short-term at -20°C until further analysis or long-term at -80°C.

Polymerase Chain Reaction (PCR)

PCR was used to amplify the mitochondrial 16S and 12S rDNA genes and the cytochrome oxidase subunit I gene (COI). Primers used are designed by Dr. Fang according to alignments of available tick full mitochondrial genome sequences.

Primers used for amplification of the 400 base pair portion of the 12S gene used in this study are: 12S aiF: AAACTAGGATTAGATACCCTATTAT and 12S biRC: AAGAGCGACGGGCGATGTGT. The 12S program was: 30 seconds at 98°C, 7 seconds at 98°C, 12 seconds at 52°C, 30 seconds at 72°C for 34 cycles, followed by 5 minutes at 72°C.

Primers used for amplification of the 523 base pair portion of the COI gene used in this study are: Tick COI 51F: 5'-ACW AAY CAT AAA GAC ATT GGD ACW ATA-3' and Tick COI 538R: 5'-GTA ATW AAW ACW GAT CAW ACA AAT AAW GGT A -3'. The COI program was: 30 seconds at 98°C, 7 seconds at 98°C, 8 seconds at 54°C, 12 seconds at 72°C for 34 cycles, followed by 5 minutes at 72°C.

Primers used to amplify the 444 base pair portion of the 16S gene region analyzed in this study are: Tick 16S 484F: 5'- TTW TWA TTW AGA TAG AAW CCA ACC TG -3' and Tick 16S 928R: 5'- GCT GTA GTA TTT TGA CTA TAC AAA GG -3'. The 16S program was: 30 seconds at 98°C, 7 seconds at 98°C, 8 seconds at 50°C, 12 seconds at 72°C for 34 cycles, followed by 5 minutes at 72°C. Each PCR reaction mixture had a volume of 25 µL and contained: 17.4 µL ddH20, 5 µL 5X buffer with $MgCl_2$, 0.5 µL dNTPs mixture, 0.5 µL of each primer (forward and reverse), 0.5 µL taqPolymerase and 1 µL of template DNA. PCR products and negative controls were visualized on 1% agarose gels and compared to a 1 kb ladder for correct band size and purity.

Data Analyses

Selected positive PCR -products were purified for DNA sequencing. The desired PCR product was re-amplified with a total volume of 50 µL. An agarose gel was then run to confirm reamplification. Purifications were done using the QIAquick PCR purification Kit or QIAquick Gel purification kit (Qiagen Inc., Valencia, CA). Purified DNA was sent to Clemson University Genomics Institute and sequenced via the Sanger sequencing method. PCR products were sequenced from both ends using the PCR primers.

Forward and reverse sequences (contigs) were assembled into consensus sequences using BioEdit. Consensus sequences were also aligned in BioEdit via CLUSTAL alignment and then gaps were rearranged by eye. Alignments were used for phylogenetic analysis using Phylogenetic Analysis Using Parsimony and others (PAUP*, Swofford).

Outgroups for analysis of all three genes were non-North American *Dermacentor* tick sequences previously deposited in GenBank. For the COI and 16S analyses, previously published North American *Dermacentor* tick sequences were downloaded and added to the alignments in order to compare findings. Downloaded published sequences are listed in Tables 5 (COI) and 6 (16S) along with their authors and accession numbers.

Maximum Parsimony (MP) analysis was performed in PAUP 4.0b10 (Swofford 2002) by using a 10,000 replicate random-addition heuristic search with branch swapping. Transitions and transversions were weighted equally, and gaps were treated as a 5th base in analyses using only original North American *Dermacentor* sequences, or as missing data in analyses in which published sequences were added to the alignment.

Maximum Likelihood (ML) analysis was also conducted using PAUP 4.0b10. The empirically derived base frequencies were used, the transition/transversion rate was estimated from the data set, and the Hasegawa-Kishino-Yano (Hasegawa et al. 1985) model was invoked.

After initial tree estimation, maximum parsimony and maximum likelihood analyses were repeated on 10,000 bootstrap replicate data sets to generate branch support values.

Results

Extraction of Nucleic Acids

We attempted to extract DNA from a total of 108 individual North American *Dermacentor* tick specimens, and were able to use a total of 86 specimens for phylogenetic analysis- which is an extraction success rate of 80%. Of the 22 specimens that failed to yield usable DNA for phylogenetic analysis, 19 had been stored for long periods of time in ethanol, 2 were immature stages stored short term in ethanol, and 1 had been stored in RNAlater solution (Qiagen). There was more variation in amplification success rates in each of the 3 genes. 12S was the first gene region attempted, and due to a large amount of nonspecific binding that required multiple purification steps, this fragment was used to a lesser extent than 16S and COI, which yielded higher success rates of received clean sequences. Numbers of each species successfully sequenced for each gene region are given in Table III.

12S

A 288 base pair region of the 12S rDNA gene was successfully sequenced from 25 individual North American *Dermacentor* ticks, representing 7 species (Table III). Of these 288 total characters, 217 were constant and 24 were parsimony uninformative, leaving 47 variable, parsimony informative sites. Figure 1 shows the majority-rule consensus tree generated via a 10,000 replicate heuristic search under maximum parsimony (MP) criterion. Bootstrap values are also based on 10,000 replicates. Figure 2 shows the majority-rule consensus tree generated via a 10,000 replicate heuristic search under Maximum Likelihood (ML) criterion (tree score =

1019.1) and bootstrap support values are based on 10,000 replicates. In both analyses, one-host *Dermacentor* species (only *D. albipictus* and *D. nitens* were included in the 12S analysis) resolved as a clade with 70% MP bootstrap support and 86% ML bootstrap support. Eastern *D. albipictus* that fit the traditional morphological description of *D. nigrolineatus* (Packard 1869) resolved as a distinct clade with 67% bootstrap support in the MP analyses (Figure 1) and 69% support in the ML analysis (Figure 2). Within these groupings, both analyses also showed clear internal groupings with high bootstrap supports, where ticks from the east coast (Georgia, Virginia, Connecticut, Maryland, and New Jersey) formed one lineage with 96% bootstrap support on the MP tree and 98% bootstrap support on the ML tree, and *D. albipictus* from the central region of the U.S. (Wisconsin, Missouri, Texas) formed the second grouping within that clade with 99% bootstrap support in both the MP and ML analyses. The *D. albipictus* specimens collected from Wyoming grouped with the single specimen of *D. nitens* with 99% bootstrap support in both analyses as well. All specimens of *D. variabilis* resolved as a monophyletic clade with 95% bootstrap support in both analyses, while also showing that *D. variabilis* from California may group more tightly together than *D. variabilis* from the eastern region of this species' range (Tennessee and Texas).

COI

For the analysis of the mitochondrial cytochrome oxidase subunit I gene that included only the sequences generated in this study, a 476 base pair region was successfully sequenced for 59 total individual *Dermacentor* ticks, representing 9 species (Tables III and IV). Of the 476 total characters, 297 were constant, and 36 variable characters were parsimony uninformative, leaving 143 parsimony informative sites. Figure 3 shows the Maximum Parsimony tree generated via a 10,000 replicate heuristic search under maximum parsimony criterion, and bootstrap support

values also represent 10,000 replicates. Figure 4 shows the Maximum Likelihood tree generated via a 10,000 replicate heuristic search under Maximum Likelihood criteria with a best tree score of 3620, with each branch showing bootstrap values obtained with 10,000 replicates as well. Outgroups for these analyses were non-North American *Dermacentor* sequences downloaded from GenBank (Table V). In both analyses, North American *Dermacentor* resolved as a monophyletic clade with 78% bootstrap support on the MP tree and 68% bootstrap support on the ML tree. One host *Dermacentor* ticks (*D. albipictus*, *D. nitens*, and *D. dissimilis* were included in this analysis) resolved as a monophyletic clade with 94% MP bootstrap support and 90% ML bootstrap support. Three species, *Dermacentor hunteri, D. occidentalis, and D. nitens,* each formed a monophyletic branch with 100% bootstrap support in both analyses. *Dermacentor variabilis* resolved as a monophyletic clade, but bootstrap support for the eastern population of *D. variabilis* was stronger than for the species as a whole. *Dermacentor andersoni* and *D. parumapertus* grouped together on a single branch with 100% MP and 99% ML bootstrap support. *Dermacentor albipictus* again showed a complex structure, with East Coast ticks fitting the morphological description of the former *Dermacentor nigrolineatus* (Packard 1869) forming a distinct clade with 99% MP and 97% ML bootstrap support, but appearing also as a sister taxon to a grouping of *D. albipictus* collected from the West, Midwest, and New Hampshire (denoted as "*D. albipictus* Lineage 2 on Figures 3 and 4) that form a group with *D. nitens* and *D. dissimilis*. A third clade of *D. albipictus* (denoted "*D. albipictus* Lineage 1" on Figures 3 and 4) groups on the other side of *D. nitens* and *D. dissimilis* on a branch with 83% MP and 89% ML bootstrap support.

COI combined analysis with published sequences

To compare the 3 distinct *D. albipictus* lineages shown in the analyses of the data generated in this study to the 2 lineages reported by Leo et al. (2010), we aligned published sequences from that study available on GenBank with sequences obtained in this study to generate 2 additional trees, one using Maximum Parsimony (Figure 5) and one using Maximum Likelihood (Figure 6). These analyses involved a 731 base pair region of the COI gene for 81 total North American *Dermacentor* tick specimens, with 59 sequences from the present study, and 22 sequences obtained from GenBank. The accession numbers and authors for these sequences are listed in Table 5. Of the 731 characters, 502 were constant and 62 variable characters were parsimony uninformative, leaving 167 parsimony informative sites. The grouping of *D. albipictus* denoted "Lineage 1" in the previous analysis grouped with all specimens that Leo et. al (2010) denoted as *D. albipictus* Lineage 1 in an internal clade with 98% MP and 95% ML bootstrap support, as part of a branch that continued to fall outside of the clade formed by the other 2 *D. albipictus* "lineages" and *D. nitens* and *D. dissimilis*. The grouping of Eastern *D. albipictus* denoted "Lineage 3" was not disrupted by any of the specimens from Leo et al. (2010) and continues to consist of the same specimens as it did in the previous COI analyses with 99% MP and 98% ML bootstrap support. However, the *D. albipictus* grouping denoted "Lineage 2" was added to by all specimens designated as Lineage 2 by Leo et al. (2010). One host *Dermacentor* continued to resolve as a monophyletic clade in both analyses, with 86% MP and 81% ML bootstrap support. *D. occidentalis*, *D. nitens*, and *D. hunteri* each formed monophyletic branches with 100% bootstrap support on both the MP and ML trees. *Dermacentor andersoni* and *D. parumapertus* together formed a single monophyletic branch with 100% bootstrap support in the MP analysis, but constituted their own branchings within a 99%

bootstrap supported clade on the ML tree, with the two specimens of *D. andersoni* forming a subclade with 62% bootstrap support, and the two *D. parumapertus* samples forming a subclade with 88% bootstrap support. North American *Dermacentor* form a monophyletic group with 78% MP and 66% ML bootstrap support.

16S

A 345 base pair region of the 16S rDNA gene was successfully sequenced from 76 individual North American *Dermacentor* ticks. Of 345 total characters, 225 characters were constant, and 10 of the variable characters were parsimony uninformative, leaving 110 variable, parsimony informative sites. Forty-one 16S rDNA sequences were downloaded from GenBank (Table VI) in order to compare our groupings within *D. albipictus* to those found in previous studies (Leo et. al 2010, Crosbie et. al 1998). A combined alignment of original and published sequences was subjected to analysis by a 10,000 replicated heuristic search under maximum parsimony (Figure 7) and maximum likelihood criteria (Figure 8). Bootstrap values are also based on 10,000 replicates for each analysis. Three *D. albipictus* groupings were observed in both analyses with sequences denoted Lineage 1 in previous analyses continuing to form a single clade (82% MP and ML bootstrap support) with all specimens designated "Lineage 1" by Leo et. al (2010) and also including the specimen called Washington-B by Crosbie et. al (1998). *D. albipictus* "Lineage 2" continued to include the same original sequences and specimens published by Leo et. al (2010) and also included two *D. albipictus* samples, one from California and one from New Mexico published by Crosbie et. al (1998) on a branch with 86% MP and ML bootstrap support. The original sequences comprising D. *albipictus* "Lineage 3" continued to form a branch with 99% MP and ML bootstrap support, but also share an 84% MP and ML bootstrap supported branch with

original sequences from Texas and Missouri, as well as *D. albipictus* specimen "Washington-B" published by Crosbie et. al (1998). As in all other analyses, *D. nitens* and *D. dissimilis* positioned with *D. albipictus* Lineage 2 and 3 on one side, and Lineage 1 on a separate branch. All remained in the one-host *Dermacentor* monophyletic branch with 94% bootstrap support on both trees. Four specimens of *Dermacentor nitens* resolved as a monophyletic species with 100% bootstrap in both analyses. Three specimens of *Dermacentor dissimilis* resolved as a monophyletic species with 81% bootstrap support on both the MP and ML trees. Both *D. nitens* and *D. dissimilis* formed a 60% bootstrap supported branch (MP and ML) with Lineage 2 and 3 of *D. albipictus*. *Dermacentor variabilis* formed a single branch with 77% bootstrap (MP and ML) support, but showed structure within the species, with ticks from the Eastern portion of its range forming a branch with 56% bootstrap (MP and ML) support, and those from the Western portion of its range (California) grouping together on a 99% bootstrap supported branch (MP and ML) within the species' clade. *Dermacentor occidentalis* resolved as a monophyletic species with 71% bootstrap support in both analyses. *Dermacentor hunteri* resolved as a single species with 98% bootstrap support on both trees. Three specimens of *D. halli* resolved as a monophyletic species with 100% bootstrap support in both the MP and ML trees. All specimens of *D. andersoni* and *D. parumapertus* again formed a monophyletic clade with 88% MP and ML bootstrap support in which specimens of each species were interspersed. All North American three-host *Dermacentor* tick species grouped together into a single clade in the larger 16S analysis with 80% bootstrap support on both the MP and ML trees. North American *Dermacentor* ticks resolved as a monophyletic group with 99% bootstrap support on both the MP and ML tree, with even the Central

American *Dermacentor* specimen of *D. imitans* (Crosbie et. al 1998) falling to the outside of this grouping.

Discussion

North American *Dermacentor*

We found considerable evidence for the monophyly of North American *Dermacentor.* Each analysis used non-North American *Dermacentor* sequences as outgroups: Palearctic *D. marginatus*, *D. reticulatus, D. nuttalli, D. silvarum*, and the Afrotropical *D. rhinocerinus.* In all analyses, without rooting, North American *Dermacentor* formed a monophyletic clade with up to 99% bootstrap support (range: 66%-99%). The Neotropical species, *D. imitans* (Venezuela) grouped outside of this clade, while the Neotropical species *Dermacentor nitens* and *Dermacentor dissimilis* both consistently grouped inside of this North American *Dermacentor* clade in every analysis. This is interesting because *D. nitens* was placed in the genus *Otocentor* by Cooley (1938), and both *D. nitens* and *D. dissimilis* formerly comprised the separate genus *Anocentor* Schulze, which was later designated as a subgenus of *Dermacentor* (Diamant and Strickland 1965). Borges et al. (1998) concluded through numerical taxonomy that *D. dissimilis* could be legitimately included within *Dermacentor*, and that *D. nitens* still formed the monotypic genus *Anocentor* and was actually more closely related to *Rhipicephalus* than to *Dermacentor*. Our data, which include all species of North American *Dermacentor* and several outgroups from non-North American ticks, strongly support the inclusion of both *D. nitens* and *D. dissimilis* as true members of *Dermacentor.*

Three-host North American *Dermacentor*

In general, the North American *Dermacentor* tick species that show high levels of host specificity, and which have relatively restricted geographical distributions, tended to resolve as

monophyletic lineages. *Dermacentor hunteri*, which parasitizes Rocky Mountain Bighorn sheep, almost exclusively resolved as a single monophyletic entity with at least 98% bootstrap support in all analyses. Individuals of *D. hunteri* processed in this study were from at least 2 distant populations of Bighorn sheep, and even when combined with the published sequences from the broad range sampling from 11 distinct populations of these host animals, done by Crosbie et al. (1998), the species continued to resolve with little to no internal structure and with high branch support. Crosbie et al. (1998) suggested that the very small amount of genetic variation observed within *D. hunteri* could be due to one or more bottleneck events. Ramey (1993) presented evidence that desert bighorn may have undergone one or more Pleistocene bottlenecks, and if *D. hunteri* has been host specific since that time, it is possible that this parasite was restricted along with its host. It is possible also, however, that existing in such a limited geographic region, and parasitizing such a narrow range of hosts, may simply provide little selection pressure for genetic evolutionary change, as a similar lack of diversity is also seen in *Dermacentor halli*, and to a lesser extent, *D. occidentalis. Dermacentor halli* is largely host specific to peccaries in its adult stage, and is present in only a few southwestern states and Mexico. Only the 16S analyses performed in this study included multiple (3) specimens of *D. halli*, but in both of the MP and ML trees, all three specimens grouped as a single monophyletic branch with 100% bootstrap support. A much larger sampling of *D. halli* is needed to determine whether it is truly as genetically homogeneous as *D. hunteri.* The Pacific Coast tick, *Dermacentor occidentalis*, has a geographic range comparable in size to that of *D. hunteri* and *D. halli*, and the 4 specimens included in COI analyses resolved as a single monophyletic entity with 100% bootstrap support. However, when twice that number of individuals were analyzed using 16S, the bootstrap support for the *D. occidentalis* branch dropped to 71%. Although this could be the result of a disparity in

variation between the COI and 16S gene regions, it is important to point out that the number of individual *D. hunteri* specimens analyzed in COI (7) nearly tripled in the 16S analysis (to 19), and the bootstrap support fell only from 100% to 98%.

Dermacentor variabilis, the American dog tick, is the most widely distributed and least host specific of the 3-host North American *Dermacentor* species, and while this species does resolve as a monophyletic entity in all analyses, it also displays more internal structure within its branch, and specifically shows divergence between members of its eastern and western populations. *Dermacentor variabilis* is found only in a small area of the far western U.S., in California and southern Oregon, and individuals collected from within this western range (California) formed an internal clade with equal (12S) or higher (16S and COI) bootstrap support than the species as a whole. *D. variabilis* is has a much larger geographical range in eastern North America, and individuals from the eastern region formed a branch with less bootstrap support than the species as a whole in all analyses.

Perhaps the most interesting genetic similarity observed in the analysis of the 3-host North American *Dermacentor* is the apparently very close relationship between *Dermacentor andersoni* and *Dermacentor parumapertus*. These two species formed a single branch with high bootstrap support (88%-100%) in both COI and 16S analyses (*D. parumapertus* was not included in the 12S analysis). In both 16S analyses, which included sequences from the Crosbie et al. (1998) study, and in the Maximum Parsimony analysis of COI, specimens of *D. parumpertus* and *D. andersoni* interspersed with one another in no apparent pattern on a single branch with 88% and 100 % bootstrap support, respectively. Crosbie et al. (1998) noted this strange relationship as well, and it is interesting to see that the inclusion of additional specimens does not help to resolve these seemingly very different species. *D. andersoni* occurs primarily at high

elevations and on a broad range of mammalian hosts and has the parallel 1st coxal spurs typical of the genus. In contrast, *D. parumapertus* occurs almost exclusively on black-tailed jackrabbits in desert and semi-desert areas in the southwestern United States, and has divergent coxal spurs (Furman and Loomis 1984). Perhaps additional genetic markers such as nuclear gene regions and microsatellite analysis could help to molecularly differentiate these species.

In both the MP and ML analyses carried out on 16S, the largest data set, all 6 North American *Dermacentor* species that employ a 3-host life cycle (*D. andersoni, D. halli, D. hunteri, D. occidentalis, D. parumapertus,* and *D. variabilis*) formed a monophyletic branch with 80% bootstrap support. This monophyly of 3-host North American *Dermacentor* is not seen in the 12S or COI analyses, and it was not observed in the 2 previous bodies of molecular phylogeny work involving New World *Dermacentor*. The 16S trees generated in this study do, however, represent the most comprehensive sampling ever done on this group of ticks, and it would be interesting to see if more specimens and more genes would further support this finding.

One-host *Dermacentor*

We found considerable support for the monophyly of one-host *Dermacentor* ticks. This study was the first to molecularly assess this group as a whole, as it was the first to include *D. dissimilis,* multiple specimens of *D. nitens*, specimens of *D. albipictus* collected from the eastern portion of its range, and specimens morphologically identified as the recently re-established species *Dermacentor kamshadalus* (Apanaskevich 2013). All one-host *Dermacentor* ticks formed a monophyletic clade, in both MP and ML analyses of all 3 mitochondrial gene regions used in this study, with 70%-94% bootstrap support. As suggested by Crosbie et al. (1998), our data support the idea that the 1-host life cycle may have evolved only once within *Dermacentor*,

in a tick ancestral to these 4 extant species. A previous study of chromosome morphology found evidence that *D. albipictus* and *D. nitens* likely share a common ancestor not shared by the 3-host species of the genus (Gunn and Hilburn 1990) and single-host specificity has been characterized as a recent evolutionary acquisition (Hoogstraal and Aeschilmann 1982). Based on this evidence, it is reasonable to assume that all North American *Dermacentor* species arose from a single common ancestor in the New World, and that a single descendant of that ancestral species evolved a one-host life cycle that gave rise to all extant one-host *Dermacentor* species, and that there was even possibly only one descendant species of that New World ancestral *Dermacentor* that gave rise to all extant North American three-host *Dermacentor* species.

The Neotropical one-host *Dermacentor* species analyzed each formed well supported monophyletic taxa. In all instances where more than one specimen of the Neotropical species *Dermacentor nitens* was analyzed (3 individuals for COI and 4 individuals for 16S), the species resolved as a single monophyletic entity with 100% bootstrap support. Only in the 12S analyses, in which only one specimen of *D. nitens* was included, did we observe the same branch sharing of this species and the Nearctic one-host species *Dermacentor albipictus* that both Crosbie et al. (1998) and Leo et al. (2010) reported. As previously mentioned, the present study is the first to include the one-host Neotropical species *Dermacentor dissimilis* in a phylogenetic analysis of this genus, and we found that *D. dissimilis* groups more tightly with *D. nitens* than does *D. albipictus*, but still does not directly share a branch with *D. nitens* in any of our analyses. Only the 16S analysis included multiple specimens of *D. dissimilis*, and all 3 formed a monophyletic clade with 81% MP and ML bootstrap support.

The Winter Tick

By far the most complex phylogeny in the New World *Dermacentor* is that of the winter tick, *D. albipictus.* The considerable phenotypic variation within this species has caused debate about its proper taxonomic position and species boundaries since it was originally described as *Ixodes nigrolineatus* and *Ixodes albipictus* by Packard in 1869 and then synonymized as only *D. albipictus* by Cooley in 1938. The form of winter tick primarily encountered in the Southeastern U.S. and along the Eastern seaboard (except in the far North-East) is the phenotype that was formerly known as *D. nigrolineatus.* This form was defined mostly by its lack of whitish pigment on the adult scutum. Although both previous molecular phylogenetic studies on *D. albipictus* reported significant molecular variation within the species, neither study included any specimens from the East. Crosbie et al. (1998) sampled only from Washington State, California, and New Mexico, and Leo et al. (2010) sampled only from Alberta. Nevertheless, both Crosbie et al. and Leo et al. reported two separate "lineages" within just the western representation of *D. albipictus,* having no clear correlation with morphological features, host associations, or geographical region. In the Crosbie et al. (1998) study, the specimen of *D. albipictus* (WA-B) that joined *D. nitens* to form a separate group away from the 3 other *D. albipictus* specimens, and which is consistently a member of the clade designated "Lineage 1" in both the Leo et al. (2010) analysis and the present study, was actually simultaneously collected from the same individual bighorn sheep in Washington State as the *D. albipictus* specimen (WA-A) that groups within the clade designated "Lineage 2" in both the Leo et al. analysis and the present study. Similarly, Leo et al. (2010) established the two molecular "lineages" referred to in that study and in this one using only samples from within Alberta, but concluded that these "deep mitochondrial DNA lineage divergences" do not indicate distinct species due to the lack of corresponding

morphometric or bacterial endosymbiont divergence, and based on the lack of divergence in the nuclear ITS-2 gene. However, none of the ticks analyzed in the Leo et al. study would have ever been identified as *D. nigrolineatus.* The sequences generated in this study also fall, seemingly randomly, into the same "Lineage 1" and "Lineage 2" discussed by Crosbie et al. (1998) and Leo et al. (2010). However, all analyses in this study establish a possibly third lineage consisting of those ticks that mostly were collected from the Eastern United States. We have designated this grouping "Lineage 3" or "Eastern *D. albipictus*" and these ticks (collected from Georgia, West Virginia, Florida, Maryland, Virginia, New Jersey, Pennsylvania, and Connecticut) formed a monophyletic clade in all analyses with 96%-99% bootstrap support. Additionally, it appears that Lineage 3 and Lineage 2 are more closely related to one another, than they are to Lineage 1, as Lineages 2 and 3 share branches in the 12S (67%-69% bootstrap support), COI (64% bootstrap support), and 16S (87% bootstrap support) analyses. Lineages 2 and 3 also appear to be more closely related to *D. nitens* and *D. dissimilis* than to Lineage 1 of *D. albipictus*, as these Neotropical one-host ticks form a clade with Lineages 2 and 3 in all analyses (54%-84% bootstrap support) that Lineage 1 is always outside of this clade.

Even the extensive and comprehensive sampling of *D. albipictus* across its range, and the use of 3 different gene regions did not resolve these two *D. albipictus* lineages, as in all analyses the sequences obtained in this study displayed an analogous paraphyly, where the branch on which a specimen fell did not seem to be readily predictable based on other factors. Lineage 1 includes *D. albipictus* collected from California, Arizona, Idaho, Washington, Mexico and Alberta. Lineage 2 includes ticks collected from Missouri, Wisconsin, Texas, New Hampshire, Idaho, Colorado, New Mexico, Washington, and Kansas, and Alberta. Additionally, some analyses indicate that these groupings are not distinct lineages, as all 3 "Lineages" discussed in

this study demonstrated paraphyly in at least one analysis. There are several instances of 2 ticks collected from the same individual animal diverging into separate lineage groups. This occurred in ticks collected from the same host animal in Kansas (D164 and D165) that grouped together as part of "Lineage 3" in the 16S analyses, but D165 grouped as part of "Lineage 2" or as a part of a paraphyletic sister taxa to Lineage 2 or Lineage 3 in the COI analyses. Another notable occurrence of this was with 3 ticks morphologically identified as the recently re-established species *Dermacentor kamshadalus*, that were all collected from the same mountain goat in Washington State (D161, D162, and D163). D161 and D162 were analyzed using COI and D161 grouped with "Lineage 1" while D162 formed a sister taxon to a paraphyletic branch of Eastern *D. albipictus*. The same paraphyly of this morphologically distinct species is observed in the analysis of D161 and D163 in under both analyses of 16S. This further illustrates the incongruence between mitochondrial molecular findings and morphological taxonomy in this group of ticks. *D. kamshadalus* is morphologically distinct from *D. albipictus*, and though both species can be found parasitizing the same individual host animal, they maintain their discrete characters and do not seem to hybridize (Apanaskevich 2013). However, under analysis of the 3 mitochondrial gene regions in this study, *D. kamshadalus* is molecularly indistinguishable from *D. albipictus* and is apparently paraphyletic in the same unpredictable manner. Additional *D. kamshadalus* specimens need to be analyzed using more genetic markers to further investigate whether its re-instatement as a species is molecularly supported.

It is possible that one or both of these convoluted lineages has an unorthodox mitochondrial genome or mitochondrial inheritance pattern due to introgression, or a similar genetic phenomenon. It is also possible that the extensive but fragmented distribution and broad

host specificity may result in the formation of multiple disjunct, isolated populations with concommitantly disrupted gene flow and subsequent population differentiation (Nadler 1995).

Implications for Molecular Identification

The original motivation for this project was the hope that a complete molecular delimitation of North American *Dermacentor* tick species would allow reliable molecular identification of each species via DNA barcoding. The immature stages of *Dermacentor* are extremely difficult to identify to the species level, which leads to frequent misidentifications and could lead to confusion about disease agent vectorship capabilities of each species. In order to create a DNA barcode for a species, it is necessary to understand exactly where species boundaries lie, as the reliability of a DNA barcode necessarily depends on variation within the barcode region that is an order of magnitude higher than the intraspecific variation within that region (Waugh 2007). The cytochrome oxidase subunit I (COI) region of the mitochondrial genome has been proposed as a potentially universal DNA barcoding region for animals (Hebert et al. 2003), and we chose this gene for phylogenetic analysis in hopes that the sequences we obtained could also serve as barcodes once all of the species were firmly delimited.

However, Leo et al. (2010) reported two deeply divergent lineages (mean difference of 7.1% for COI and 4.5% for 16S) in morphologically and ecologically indistinguishable populations of *D. albipictus,* that would normally be considered diagnostic of distinct species in DNA barcoding studies. This means that *D. albipictus* Lineage 1 and Lineage 2 would have to be barcoded as separate species. *Dermacentor* is considered to be one of the most recently derived ixodid genera (Oliver 1989), and we conclude that mitochondrial barcodes may not be a feasible identification technique within this genus at the current stage of our knowledge.

More work on systematics of North American *Dermacentor* using morphological and molecular techniques is needed, particularly within *D. albipictus.*

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Table I. Species abbreviations used in figures

Species	Abbreviation
Dermacentor albipictus	ALB
Dermacentor andersoni	AND
Dermacentor dissimilis	DIS
Dermacentor halli	HAL
Dermacentor hunteri	HUN
Dermacentor nitens	NIT
Dermacentor occidentalis	OCC
Dermacentor parumapertus	PAR
Dermacentor variabilis	VAR

Table II. Locality abbreviations used in figures

Locality	Abbreviation
California	ca
Canada	can
Connecticut	ct
Colorado	co
El Salvador	es
Florida	fl
Georgia	ga
Guatemala	gu
Idaho	id
Kansas	ks
Maryland	md
Massachusetts	ma
Mexico	mx
Missouri	mo
Montana	mt
New Hampshire	nh
New Jersey	nj
New York	ny
North Carolina	nc
Ohio	oh
Panama	ps
Pennsylvania	pa
Tennessee	tn
Texas	tx
Utah	ut
Virginia	va
Washington	wa
West Virginia	wv
Wisconsin	wi
Wyoming	wy

Table III. Number of successful sequences of each mitochondrial gene region obtained in this study for each North America *Dermacentor* species

Dermacentor	**16S**	**COI**	**12S**
albipictus	36	38	16
andersoni	4	2	1
dissimilis	3	1	0
halli	2	1	1
hunteri	8	7	1
kamshadalus	2	2	0
nitens	3	2	1
occidentalis	5	4	1
parumapertus	2	2	0
variabilis	11	5	4
Total	76	59	25

Table IV. List of sequences obtained in this study included in analysis of each of 3 gene regions

Sample ID	Species	Location	Source	COI	16S	12S
D5	*D. andersoni*	Montana	USNTC	X	X	X
D13E	*D. variabilis*	Florida	USNTC		X	
D13G	*D. variabilis*	Florida	USNTC		X	
D14A	*D. variabilis*	Tennessee	USNTC		X	X
D14B	*D. variabilis*	Tennessee	USNTC		X	
D15B	*D. variabilis*	Ohio	USNTC		X	
D16A	*D. variabilis*	Kansas	USNTC		X	
D19	*D. albipictus*	Arizona	USNTC	X	X	X
D20	*D. albipictus*	California	USNTC	X		X
D23	*D. albipictus*	Connecticut	USNTC	X		X
D24	*D. albipictus*	Georgia	USNTC	X	X	X
D25	*D. albipictus*	Maryland	USNTC	X	X	X
D26	*D. albipictus*	Missouri	USNTC	X	X	X
D27	*D. albipictus*	New Jersey	USNTC	X	X	X
D28	*D. albipictus*	North Carolina	USNTC			X
D30	*D. albipictus*	Virginia	USNTC		X	X
D31	*D. albipictus*	Wisconsin	USNTC			X
D36A	*D. albipictus*	Texas	Martin	X		
D36B	*D. albipictus*	Texas	Martin	X	X	X
D37A	*D. albipictus*	Texas	A. Zambrano	X	X	
D37B	*D. albipictus*	Texas	A. Zambrano	X	X	
D38A	*D. albipictus*	Mexico	V. Muniz	X	X	
D38B	*D. albipictus*	Mexico	V. Muniz	X	X	
D39A	*D. albipictus*	California	J. Mertins	X		
D39B	*D. albipictus*	California	J. Mertins	X	X	X
D39C	*D. albipictus*	California	J. Mertins		X	X
D40B	*D. occidentalis*	California	J. Mertins		X	
D41	*D. halli*	Texas	J. Mertins	X	X	X
D42	*D. occidentalis*	California	J. Mertins	X	X	
D43	*D. occidentalis*	California	J. Mertins	X	X	X
D44	*D. halli*	Texas	J. Mertins		X	
D45	*D. variabilis*	Massachusetts	D. Epstein	X	X	
D50	*D. albipictus*	West Virginia	USNTC		X	
D51	*D. albipictus*	Maryland	USNTC	X		
D52	*D. albipictus*	Maryland	USNTC	X	X	X
D53	*D. albipictus*	Missouri	USNTC	X	X	X
D57	*D. dissimilis*	El Salvador	USNTC		X	
D61	*D. albipictus*	Wyoming	USNTC			X
D66	*D. albipictus*	Florida	USNTC		X	
D67	*D. nitens*	Texas	M.S. Mesa	X		X
D68	*D. dissimilis*	El Salvador	R.P. Eckerlin		X	
D69	*D. parumapertus*	Utah	C.R. Baird		X	
D72	*D. hunteri*	Arizona	R. Babb	X	X	X
D73	*D. hunteri*	Arizona	R. Babb	X	X	
D74	*D. hunteri*	Arizona	R. Babb	X	X	

D75	*D. hunteri*	Arizona	R. Babb		X	
D76	*D. hunteri*	Arizona	R. Babb		X	
D78	*D. hunteri*	Arizona	B. Henry	X	X	
D79	*D. hunteri*	Arizona	B. Henry	X	X	
D80	*D. hunteri*	Arizona	B. Henry	X		
D81	*D. hunteri*	Arizona	B. Henry	X	X	
D84F	*D. variabilis*	California	J. Kleinjan	X	X	X
D84M	*D. variabilis*	California	J. Kleinjan		X	X
D85F	*D. occidentalis*	California	J. Kleinjan	X	X	
D85M	*D. occidentalis*	California	J. Kleinjan	X	X	
D88	*D. andersoni*	Montana			X	
D89	*D. albipictus*	Pennsylvania			X	
D91	*D. albipictus*	New Mexico			X	
D93	*D. albipictus*	Virginia			X	
D94	*D. variabilis*	Florida		X	X	
D119	*D. albipictus*	Idaho		X	X	
D120	*D. albipictus*	Idaho		X	X	
D121	*D. albipictus*	Idaho		X	X	
D122	*D. albipictus*	Idaho		X		
D123	*D. albipictus*	Idaho		X	X	
D124	*D. albipictus*	Idaho			X	
D125	*D. dissimilis*	Guatemala		X	X	
D127	*D. nitens*	Panama		X		
D161	*D. kamshadalus*	Washington		X	X	
D162	*D. kamshadalus*	Washington		X		
D163	*D. kamshadalus*	Washington			X	
D164	*D. albipictus*	Kansas		X	X	
D165	*D. albipictus*	Kansas		X	X	
D178	*D. albipictus*	Colorado		X	X	
D180	*D. albipictus*	Colorado		X	X	
D181	*D. albipictus*	Colorado			X	
D182	*D. parumapertus*	Utah		X	X	
D184	*D. andersoni*	Canada		X	X	
D186	*D. albipictus*	Georgia		X		
D187	*D. albipictus*	New York		X		
D197	*D. albipictus*	New Hampshire		X		
D198	*D. albipictus*	New Hampshire		X		

Table V. Published sequences downloaded from GenBank used in COI analysis

ID	Species	Accession #	Author
Leo 1.1	*Dermacentor albipictus*	GU968826	Leo et al. 2010
Leo 1.2	*Dermacentor albipictus*	GU968827	Leo et al. 2010
Leo 1.3	*Dermacentor albipictus*	GU968828	Leo et al. 2010
Leo 1.4	*Dermacentor albipictus*	GU968829	Leo et al. 2010
Leo 2.1	*Dermacentor albipictus*	GU968830	Leo et al. 2010
Leo 2.2	*Dermacentor albipictus*	GU968831	Leo et al. 2010
Leo 2.3	*Dermacentor albipictus*	GU968832	Leo et al. 2010
Leo 2.4	*Dermacentor albipictus*	GU968833	Leo et al. 2010
Leo 2.5	*Dermacentor albipictus*	GU968834	Leo et al. 2010
Leo 2.6	*Dermacentor albipictus*	GU968835	Leo et al. 2010
Leo 2.7	*Dermacentor albipictus*	GU968836	Leo et al. 2010
Leo 2.8	*Dermacentor albipictus*	GU968837	Leo et al. 2010
Leo 2.9	*Dermacentor albipictus*	GU968838	Leo et al. 2010
Leo 2.10	*Dermacentor albipictus*	GU968839	Leo et al. 2010
Leo 2.11	*Dermacentor albipictus*	GU968840	Leo et al. 2010
Leo 2.12	*Dermacentor albipictus*	GU968841	Leo et al. 2010
Leo 2.13	*Dermacentor albipictus*	GU968842	Leo et al. 2010
Leo 2.14	*Dermacentor albipictus*	GU968843	Leo et al. 2010
N/A	*"Anocentor" nitens*	AY008679	Murrell et al. 2001
N/A	*Dermacentor variabilis*	AF132831	Murrell et al. 2000
Outgroup	*Dermacentor marginatus*	AF132828	Murrell et al. 2000
Outgroup	*Dermacentor reticulatus*	AF132829	Murrell et al. 2000
Outgroup	*Dermacentor rhinocerinus*	AF132830	Murrell et al. 2000

Table VI. Published sequences downloaded from GenBank used in 16S analyses

ID	Species	Accession Number	Author
Leo "Lineage 1"	*Dermacentor albipictus*	GU968848	Leo et al. 2010
Leo 2.1	*Dermacentor albipictus*	GU968849	Leo et al. 2010
Leo 2.2	*Dermacentor albipictus*	GU968850	Leo et al. 2010
Leo 2.3	*Dermacentor albipictus*	GU968851	Leo et al. 2010
Leo 2.4	*Dermacentor albipictus*	GU968852	Leo et al. 2010
Leo 2.5	*Dermacentor albipictus*	GU968853	Leo et al. 2010
Leo 2.6	*Dermacentor albipictus*	GU968854	Leo et al. 2010
Leo 2.7	*Dermacentor albipictus*	GU968855	Leo et al. 2010
Leo 2.9	*Dermacentor albipictus*	GU968856	Leo et al. 2010
Leo 2.10	*Dermacentor albipictus*	GU968857	Leo et al. 2010
Leo 2.11	*Dermacentor albipictus*	GU968858	Leo et al. 2010
Leo 2.12	*Dermacentor albipictus*	GU968859	Leo et al. 2010
Leo 2.13	*Dermacentor albipictus*	GU968860	Leo et al. 2010
Leo 2.14	*Dermacentor albipictus*	GU968861	Leo et al. 2010
CroALB-WA-A	*Dermacentor albipictus*	AF001232	Crosbie et al. 1998
CroALB-WA-B	*Dermacentor albipictus*	AF001233	Crosbie et al. 1998
CroALB-CA	*Dermacentor albipictus*	AF001231	Crosbie et al. 1998
CroALB-NM	*Dermacentor albipictus*	AF001230	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001246	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001245	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001244	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001243	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001242	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001241	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001240	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001239	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001238	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001237	Crosbie et al. 1998
CroHUN	*Dermacentor hunteri*	AF001236	Crosbie et al. 1998
CroVAR1	*Dermacentor variabilis*	AF001257	Crosbie et al. 1998
CroVAR2	*Dermacentor variabilis*	AF001256	Crosbie et al. 1998
CroPAR1	*Dermacentor parumapertus*	AF001255	Crosbie et al. 1998
CroPAR2	*Dermacentor parumapertus*	AF001254	Crosbie et al. 1998
CroPAR3	*Dermacentor parumapertus*	AF001253	Crosbie et al. 1998
CroOCC1	*Dermacentor occidentalis*	AF001252	Crosbie et al. 1998
CroOCC2	*Dermacentor occidentalis*	AF001251	Crosbie et al. 1998
CroOCC3	*Dermacentor occidentalis*	AF001250	Crosbie et al. 1998
CroNIT	*Dermacentor nitens*	AF001249	Crosbie et al. 1998
CroImitans	*Dermacentor imitans*	AF001247	Crosbie et al. 1998
CroAND1	*Dermacentor andersoni*	AF001235	Crosbie et al. 1998
CroAND2	*Dermacentor andersoni*	AF001234	Crosbie et al. 1998
CroHAL	*Dermacentor halli*	AF001247	Crosbie et al. 1998
Outgroup	*Dermacentor marginatus*	JX051094	Lv et al. 2013
Outgroup	*Dermacentor nuttalli*	JX051099	Lv et al. 2013
Outgroup	*Dermacentor reticulatus*	JF928493	Karger et al. 2012

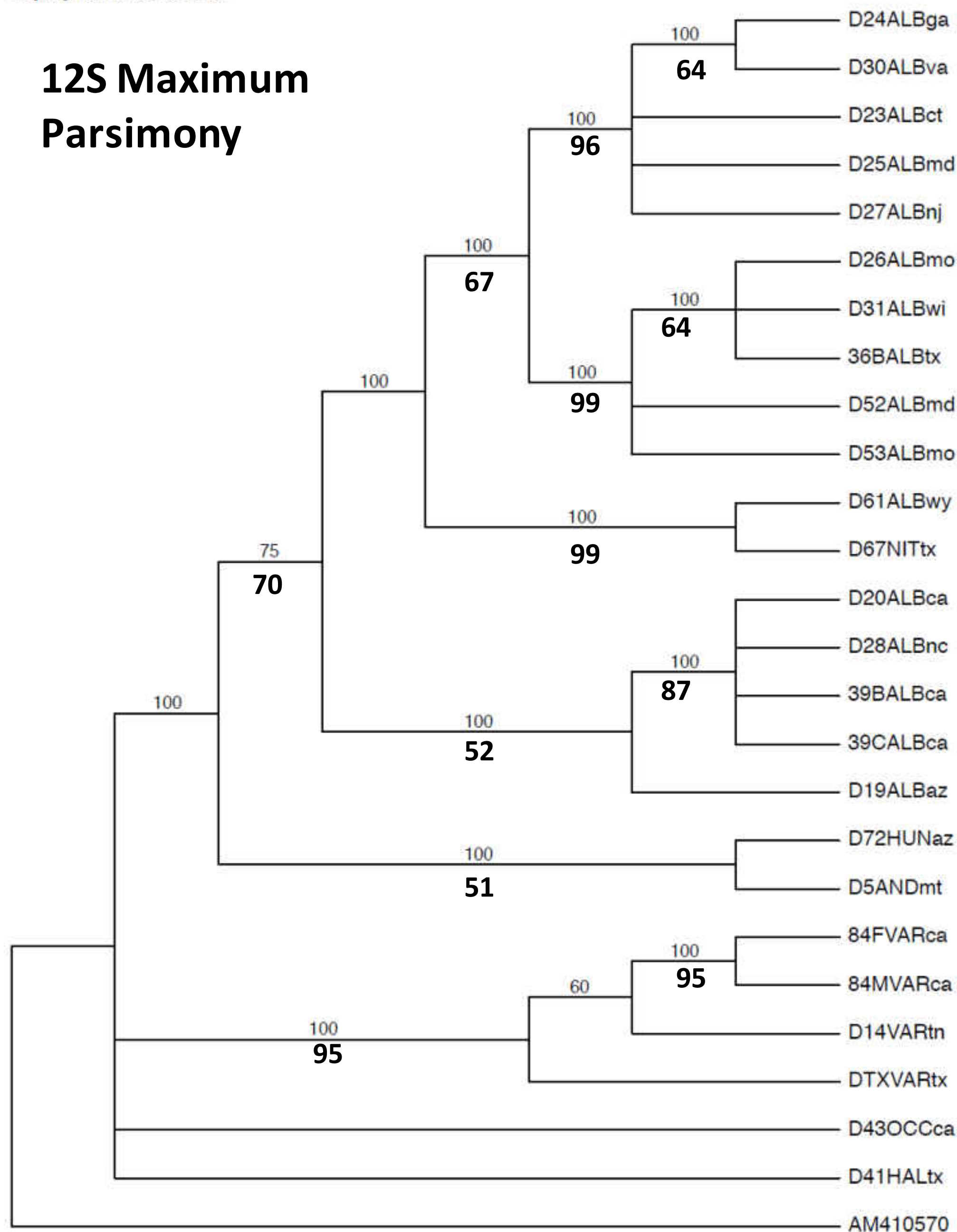

Figure 1. Mitochondrial 12S rDNA Maximum Parsimony (MP) majority rule consensus tree for North American *Dermacentor* tick specimens. Numbers above branches give percentage of generated trees that agree with this topology, and numbers below branches represent bootstrap support values based on 10,000 replicates. Species abbreviations are given in Table 3. Locality abbreviations are given in Table 4. The outgroup is a published *Dermacentor marginatus* sequence from GenBank (Accession #: AM410570).

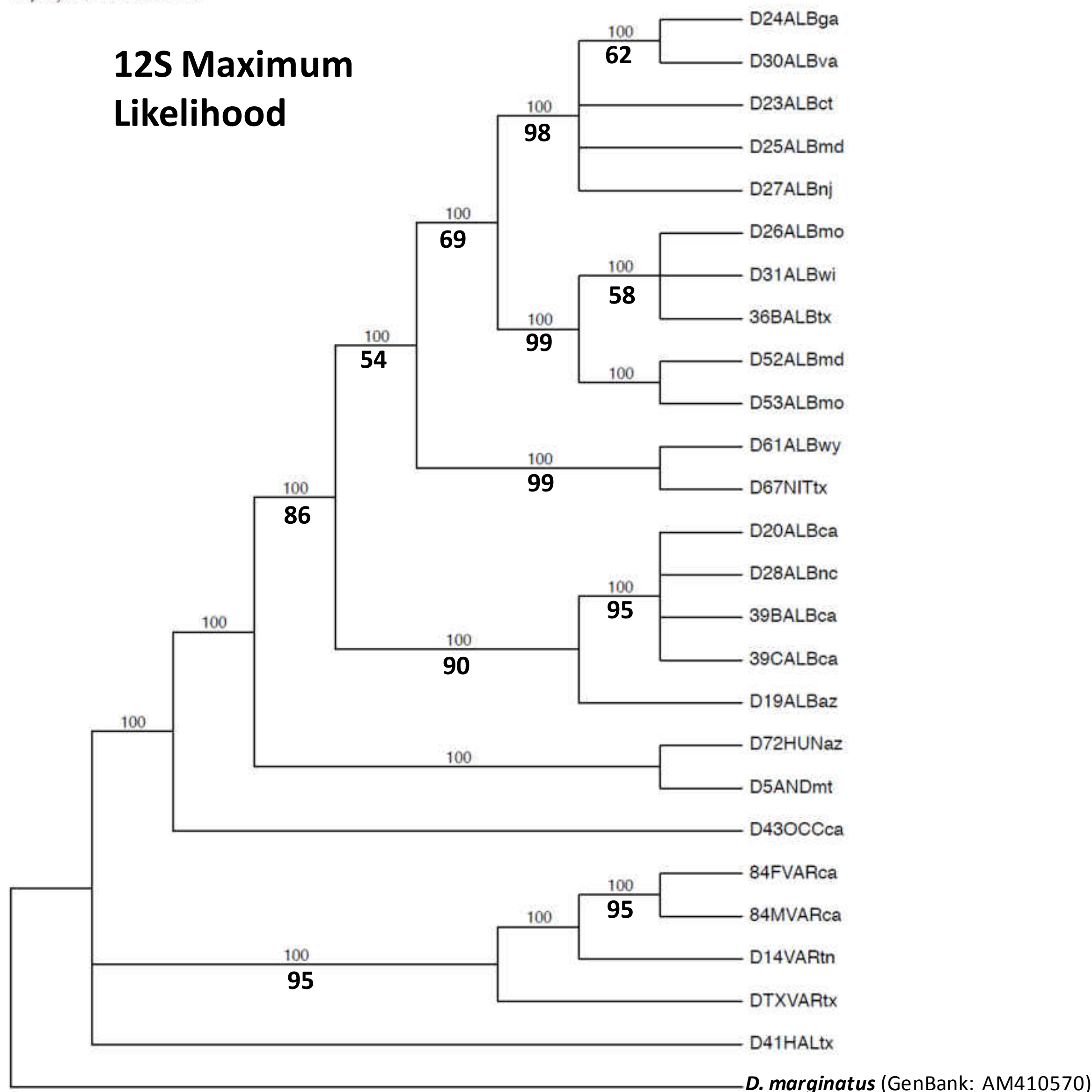

Figure 2. Mitochondrial 12S rDNA Maximum Likelihood (ML) majority rule consensus tree for North American *Dermacentor* tick specimens. Numbers above branches give percentage of generated trees that agree with this topology, and numbers below branches represent bootstrap support values based on 10,000 replicates. Species abbreviations are given in Table 3. Locality abbreviations are given in Table 4. The outgroup is a published *Dermacentor marginatus* sequence from GenBank (Accession #: AM410570).

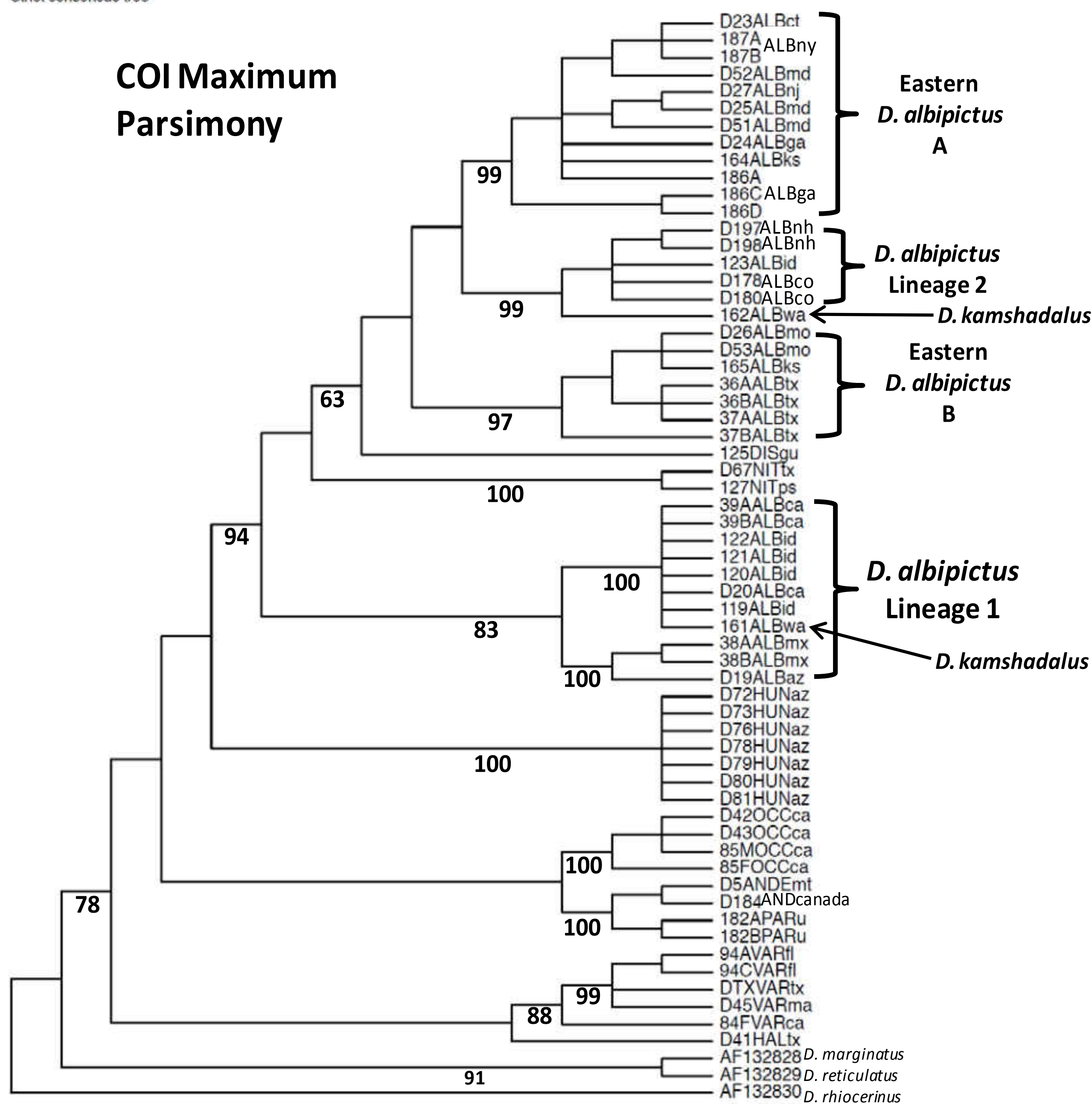

Figure 3. Mitochondrial COI Maximum Parsimony (MP) strict consensus tree for only the North American *Dermacentor* tick sequences generated in this study. Numbers below branches represent bootstrap support values based on 10,000 replicates. Species abbreviations are given in Table 3. Locality abbreviations are given in Table 4. The outgroups are published non-North American *Dermacentor* sequences from GenBank. Outgroup details can be found in Table 5.

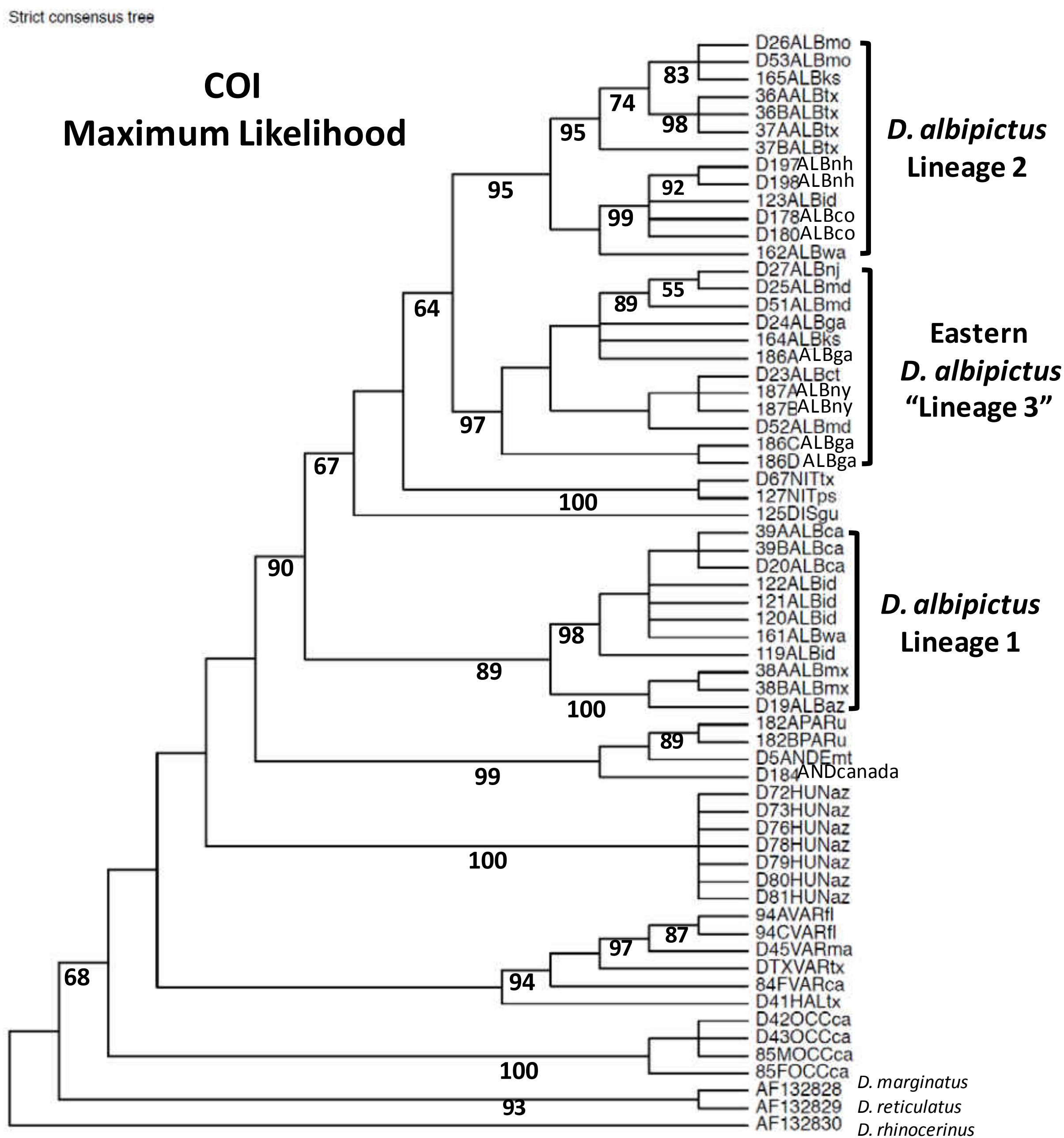

Figure 4. Mitochondrial COI Maximum Likelihood (ML) strict consensus tree for only the North American *Dermacentor* tick sequences generated in this study. Numbers below branches represent bootstrap support values based on 10,000 replicates. Species abbreviations are given in Table 3. Locality abbreviations are given in Table 4. The outgroups are published non-North American *Dermacentor* sequences from GenBank, and outgroup details can be found in Table 5.

Strict consensus tree

D. rhinocerinus
D. reticulatus
D. marginatus

COI Maximum Parsimony: joint analysis with published sequences

Maryland (D25,D51,D52),
Georgia (D186A, D186C,D186D,D24),
New York (D187A,D187B),
New Jersey (D27),
Connecticut (D23)
Kansas (D164)

Eastern
D. albipictus
A

Leo et. al "Lineage 2" (2.1-2.13)
Idaho (D123),
Colorado (D178, D180),
New Hampshire (D197, D198)
Washington (D162)

D. albipictus
Lineage 2

D. kamshadalus (D162)

Missouri (D26,D53),
Texas (D36A,D36B,D37A,D37B),
Kansas (D165)

Eastern
D. albipictus
B

D. nitens
D. dissimilis

Mexico (D38A,D38B), Arizona (D19)

Leo et. al "Lineage 1" (1.1-1.4)
California (D39A,D39B,D20),
Idaho (D119,D120,D121,D122),
D. kamshadalus (D161)

D. albipictus
Lineage 1

D. andersoni and D. parumapertus
D. occidentalis
D. variabilis
D. hunteri
D. halli

99 77 98 84 100 86 100 98 63 100 100 89 100 78

Figure 5. Mitochondrial COI Maximum Parsimony (MP) strict consensus tree for the North American *Dermacentor* tick sequences generated in this study with *D. albipictus* sequences published by Leo et al. (2010) included. Specimen details and accession numbers for sequences downloaded from GenBank can be found in Table 5. Numbers below branches represent bootstrap support values based on 10,000 replicates. The outgroups are published non-North American *Dermacentor* sequences from GenBank. Outgroup details can be found in Table 5.

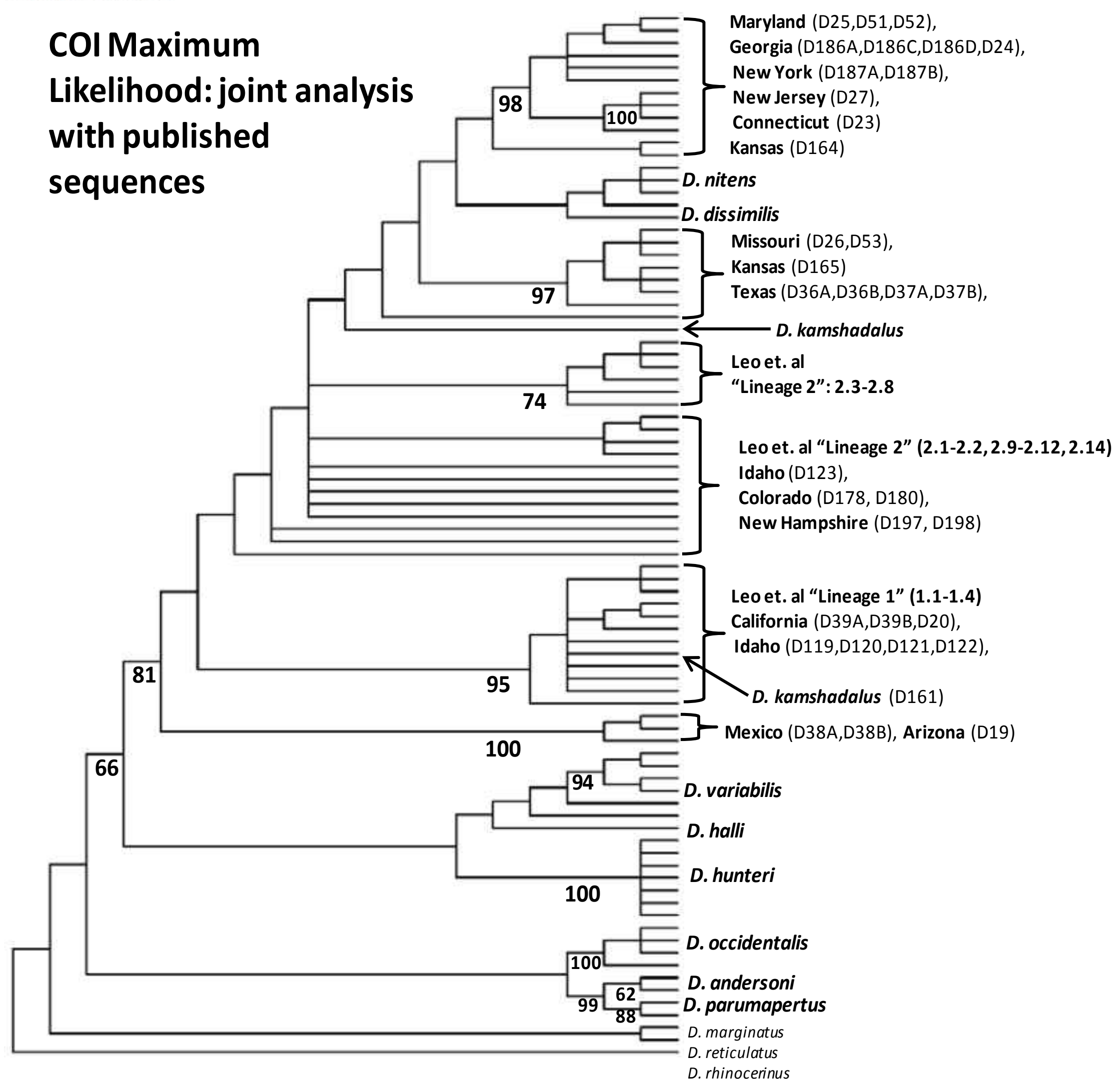

Figure 6. Mitochondrial COI Maximum Likelihood (ML) strict consensus tree for the North American *Dermacentor* tick sequences generated in this study with *D. albipictus* sequences published by Leo et al. (2010) included. Specimen details and accession numbers for sequences downloaded from GenBank can be found in Table 5. Numbers below branches represent bootstrap support values based on 10,000 replicates. The outgroups are published non-North American *Dermacentor* sequences from GenBank. Outgroup details can be found in Table 5.

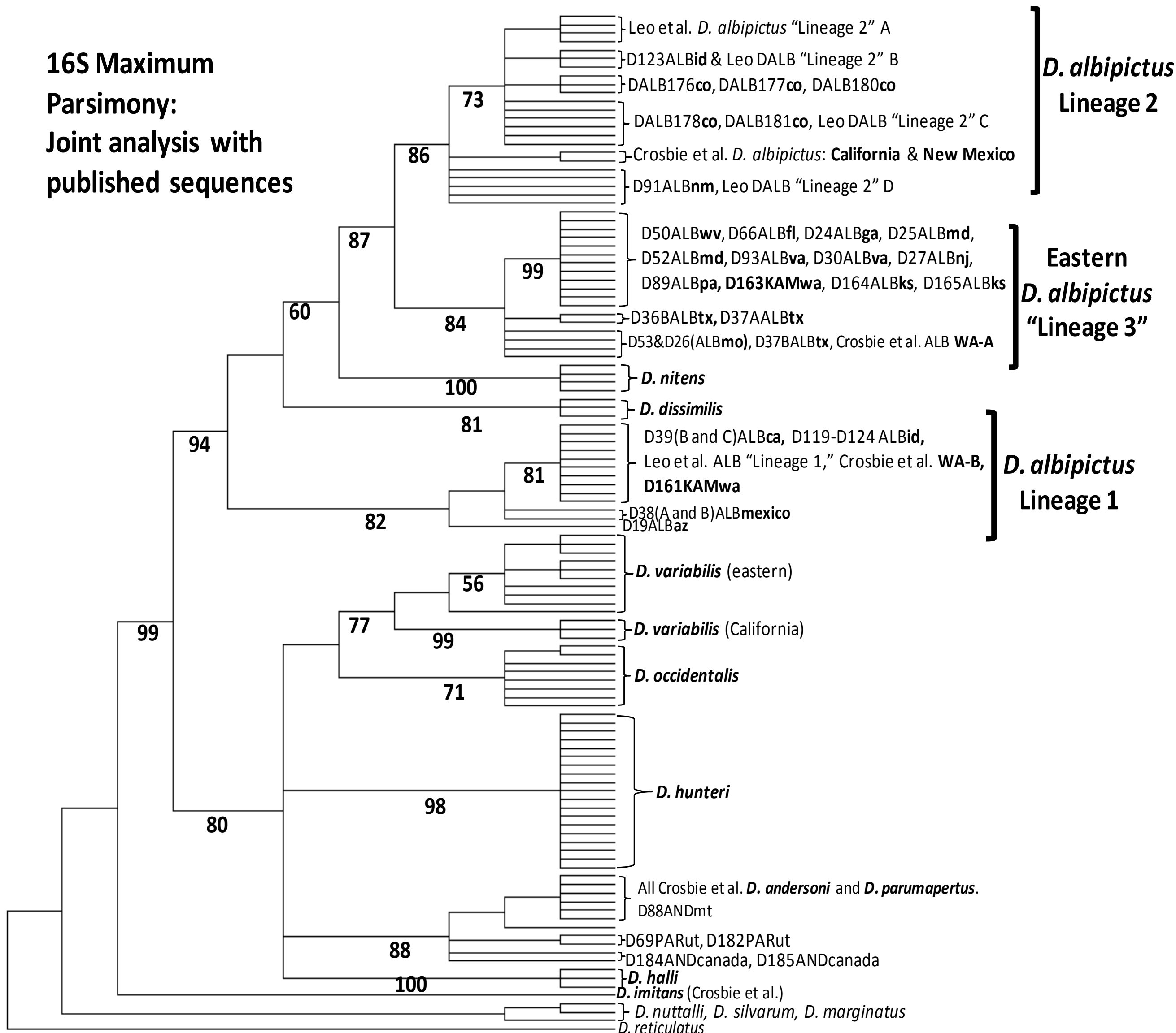

Figure 7. Mitochondrial 16S rDNA Maximum Parsimony (MP) strict consensus tree for North American Dermacentor ticks with sequences generated in this study combined with published sequences (Leo et al. 2010, Crosbie et al. 1998) with bootstrap support values indicated under each branch (10,000 replicates). GenBank accession numbers for published sequences included in this analysis are provided in Table 6.

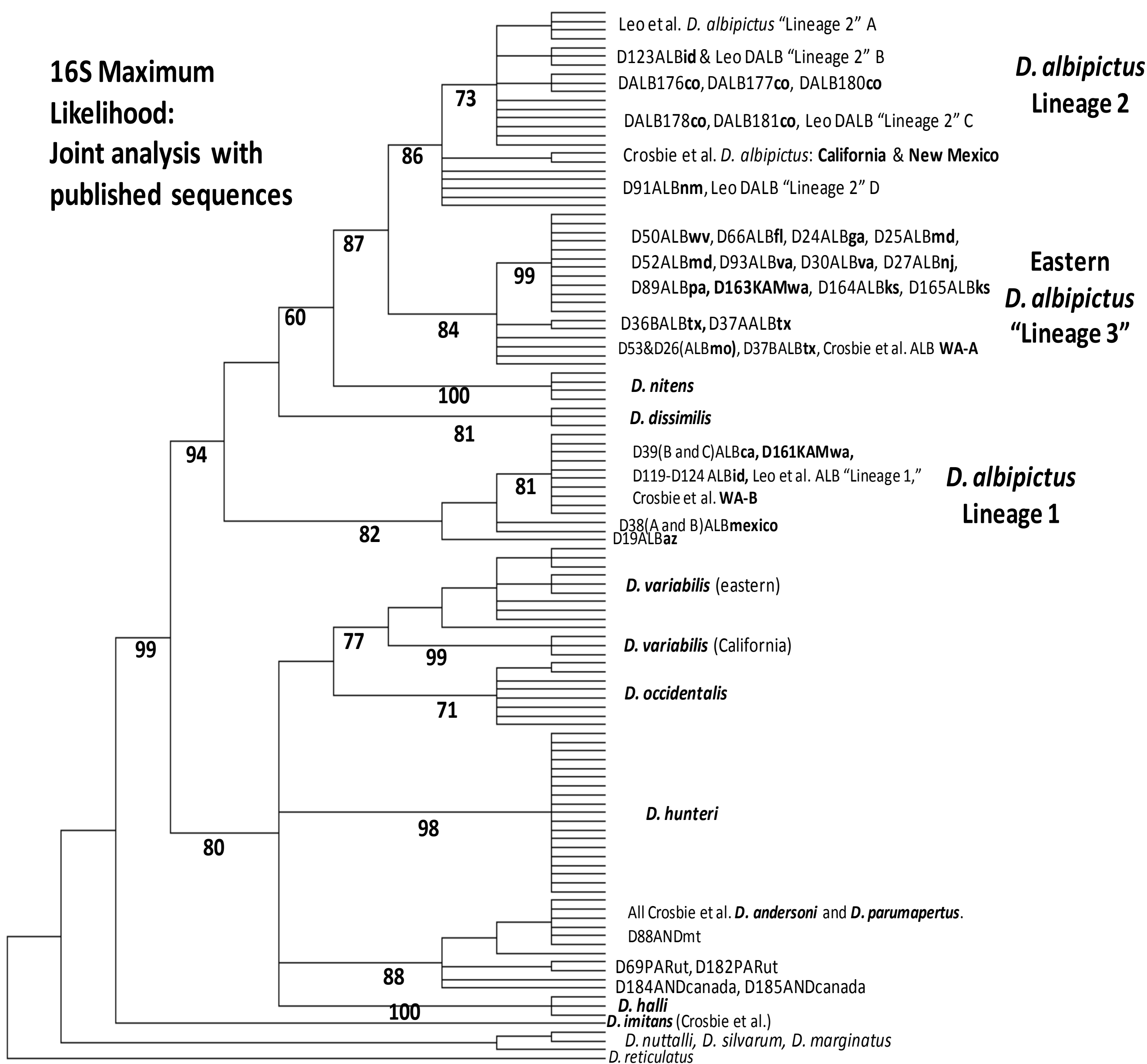

Figure 8. Mitochondrial 16S rDNA Maximum Parsimony (MP) strict consensus tree for North American Dermacentor ticks with sequences generated in this study combined with published sequences (Leo et al. 2010, Crosbie et al. 1998) with bootstrap support values indicated under each branch (10,000 replicates). GenBank accession numbers for published sequences included in this analysis are provided in Table 6.

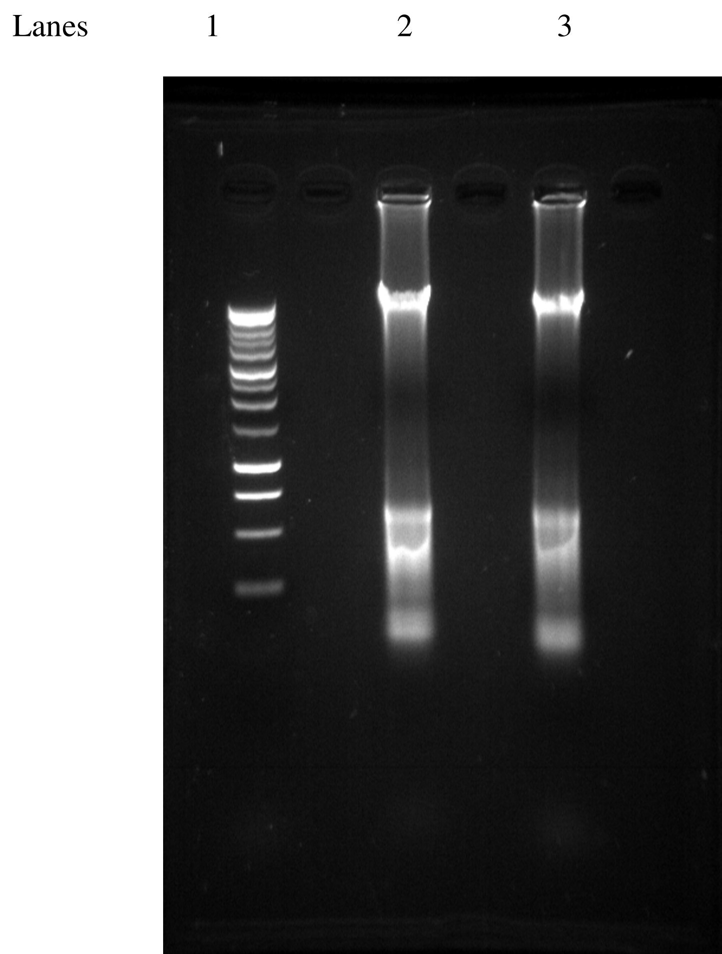

Figure 9. Agarose gel of total nucleic acid extraction from live *Dermacentor albipictus* specimens. These specimens were fresh, never subjected to ethanol storage, and yielded the highest quality nucleic acid extractions in this study. Lane 1: 1kb ladder, Lane 2: extraction product from sample D176, Lane 3: extraction product from sample D177.

Lanes 1 2 3

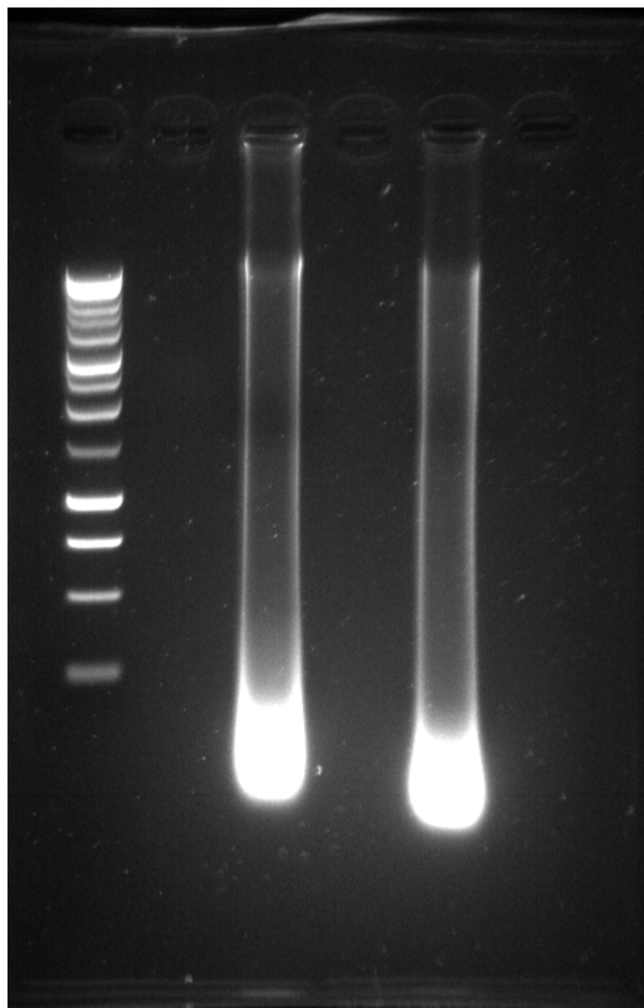

Figure 10. Agarose gel of total nucleic acid extraction from *Dermacentor albipictus* specimens that had been stored in ethanol for 1 year. Lane 1: 1kb ladder, Lane 2: extraction product from sample D197, Lane 3: extraction product from sample D198

Lanes 1 2 3 4 5 6

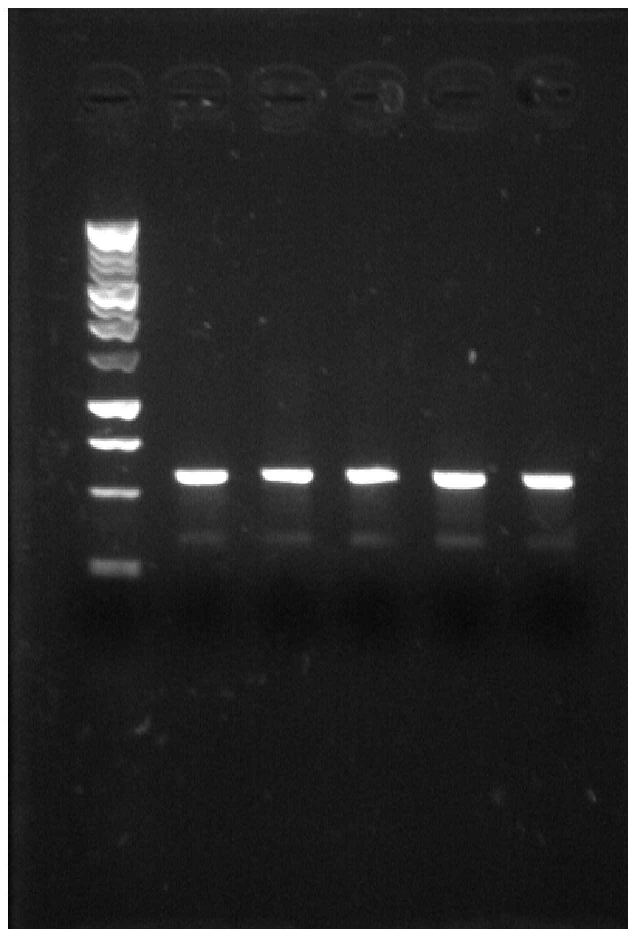

Figure 11. Agarose gel of North American *Dermacentor* PCR amplification of mitochondrial COI DNA. Lane 1: 1kb ladder, Lanes 2-6: positive samples.