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Systematics and population structure of Amblyomma maculatum group ticks and Rickettsia parkeri, an emerging human pathogen in southern Arizona, USA

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Systematics and population structure of *Amblyomma maculatum* group ticks and *Rickettsia parkeri*, an emerging human pathogen in southern Arizona, USA

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The recent discovery of *Amblyomma maculatum* sensu lato (s. l.) ticks in southern Arizona has renewed discussions around species designations for members of the *Amblyomma maculatum* tick group. *Amblyomma maculatum* s. l. from Arizona appear to be morphologically intermediate between *A. maculatum* sensu stricto (s. s.) and *A. triste* s. s. At present there is no conclusive species designation for the ticks from Arizona. My research focused on analyzing the systematics of both *A. maculatum* s. l. and *Rickettsia parkeri,* a common bacterial pathogen transmitted by these ticks.

In the laboratory, *A. maculatum* s. l. from Arizona and *A. maculatum* s. s. from Georgia readily mated on experimental animals to produce F1 hybrid ticks; there was no difference in fertility with these two populations when compared with homologous populations. However, the F1 hybrids produced during these experiments exhibited diminished fitness and did not produce a viable F2 generation. These results suggest that *A. maculatum* s. l. and *A. maculatum* s. s. represent separate biological species.

Results of the crossbreeding experiment conflict with recent genetic analyses of *A. maculatum* s. l. and *A. maculatum* s. s. suggesting they are a single species. Thus, I developed

and optimized 14 microsatellite loci that amplify both *A. maculatum* s. s. and *A. maculatum* s. l. These novel microsatellite markers can be used in future analyses of *A. maculatum* s. l. and *A. maculatum* s. s. to further test for conspecificity between the two.

I also investigated the genetic relationships within geographically distinct *R. parkeri* strains through development and implementation of a multi-locus sequence typing analysis. I showed that while there is no consistent genetic delineation of strains isolated from *A. maculatum* s. l. versus *A. maculatum* s. s., there is a subset of *R. parkeri* strains from *A. maculatum* s. l. that appear to represent an intermediate genotype between the North and South American strains. While the biological causes for these results are not immediately clear, coevolution of *R. parkeri* and *A. maculatum* s. l. may account for the detection of the intermediate genotype only found in association with *A. maculatum* s. l.

DEDICATION

This dissertation is dedicated to my children, as every good thing I've done in my life has been for them. My sweet Bee, my favorite person in the world, you made me a mother and changed the course of my life forever. This dissertation is for you – to show you that you can never dig yourself into a hole so deep that you can't climb back out. Even if you move an inch at a time, just keep moving and eventually you will find yourself in the light. To my kind, wonderful Graham(y), the greatest joy of my life, this dissertation is for you. To show you what can happen if you keep cheering for those you love. To both of you: never stop learning. Spend the rest of your lives challenging yourselves to try new things, to investigate what interests you, and to never be afraid to fail in those endeavors. The only true failure is stagnation. And always remember, success is just outside your comfort zone.

Love, Mom.

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To the rest of my committee, thank you for helping guide me through this journey. Your suggestions, comments, and scrutiny contributed to both this dissertation and my success as a graduate student. I would also like to acknowledge the Division of Vector-Borne Diseases and the Rickettsial Zoonoses Branch at CDC for their support and investment in this PhD. The CDC NCEZID long-term education (LTE) program was integral to my success, so I would like to specifically thank my committee member and supervisor Dr. Christopher Paddock for his efforts to help me get into the LTE program as well as his continued professional advocacy for me.

Finally, I would like to acknowledge my family, both blood and chosen. Thank you for your support over the last five years. You have all worked to ensure that I could always be where I needed to be and could get a break when I so desperately needed it. To Luke, Kathy, Paul, Sallie, John, Wendy, Tracy, Alyssa, Jessica, and Aubree: thank you. In many ways, this PhD is as much yours as it is mine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

A REVIEW OF LITERATURE AND BACKGROUND INFORMATION ON THE *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE) GROUP

Background and history

Ticks are obligatory hematophagous ectoparasites and are some of the most common vectors of human pathogens; worldwide, they are second only to mosquitoes in frequency of disease transmission. They exist on every continent and have been shown to parasitize every class of vertebrates. Ticks can harm their human and animal hosts through direct mechanical damage from and allergic reactions to tick feeding for bloodmeal acquisition, as well as through transmission of pathogens during feeding. Ticks carry and transmit a multitude of pathogens including bacteria, viruses, and protozoa. Tick-borne illnesses are primarily zoonotic and involve a series of complex ecological relationships, including among pathogens and their vertebrate and invertebrate hosts, between humans and animal reservoirs of zoonotic pathogens, and between vectors and their ecological niches.

Taxonomically, ticks are mites and are arthropods located in the subclass Acari, order Parasitiformes. There are approximately 900 species of ticks worldwide (Guglielmone et al. 2010) divided into two major families: Ixodidae (hard ticks) and Argasidae (soft ticks) (Anderson 2002). A third family of ticks, Nuttalliellidae, consists of a single species, *Nuttalliella namaqua*, found only in regions of southern Africa (Mans et al. 2011). This species is evolutionarily the most basal lineage of these arthropods. While both argasid and ixodid ticks

play a role in the transmission of human pathogens worldwide, ixodid ticks are the most medically important vectors of human disease in the United States. Approximately 40,000 – 50,000 cases of tick-borne diseases are reported each year; however, some estimates show that the actual case number may be closer to 300,000 (Hinckley et al. 2014, Nelson et al. 2015).

The genus *Amblyomma* Koch is a group of ixodid ticks that are primarily found in tropical and sub-tropical regions of the world, though many species are well established in the Nearctic. They are three-host ticks and are well known for their ornate scutum, long mouthparts, and presence of eyes and festoons. Species within *Amblyomma* are incredibly important worldwide as vectors of many zoonotic and veterinary pathogens. For example, in the United States, *Amblyomma americanum* is one of the most commonly encountered tick species and is the vector of many viral and bacterial pathogens (Stromdahl and Hickling 2012, Springer et al. 2014).

Taxonomy of the *Amblyomma maculatum* group

The ixodid tick *Amblyomma maculatum* Koch, 1844 (the Gulf Coast tick) is a species in the subgenus *Anastosiella* Santos Dias, 1963 (Estrada-Peña et al. 2005). First described in 1844 by the German entomologist Carl Ludwig Koch (Koch 1844, Kohls 1956), the type specimen of the Gulf Coast tick was collected in "Carolina" in the United States. Koch went on to describe five other species of *Amblyomma* from South America that same year (*A. rubripes* Koch, 1844, *A. ovatum* Koch, 1844, *A. ovale* Koch, 1844, *A. tigrinum* Koch, 1844, and *A. triste* Koch, 1844), all of which were later believed by various taxonomists to be synonyms of *Amblyomma maculatum*. These ticks were subsequently grouped together to form the *Amblyomma maculatum* tick group.

Subsequent analysis identified *A. rubripes* as a synonym of *A. maculatum*, and further work in the 1950's on these various *Amblyomma* tick species combined *A. ovatum* with *A. tigrinum*, collapsing the group from six species to four (Kohls 1956). This remained the case until 1998 when Camicas et al. reevaluated the *A. maculatum* tick group and determined that it consisted of *A. maculatum*, *A. triste*, *A. tigrinum*, *Amblyomma parvitarsum* Neumann, 1901, and *Amblyomma neumanni* Ribaga, 1902 (Camicas et al. 1998). This taxonomically dynamic tick group was subjected to yet another analysis by Estrada-Peña et al. in 2005, when advances in molecular phylogenetics were combined with traditional morphological analyses to better clarify the systematics of the group. As a result, since 2005, the *Amblyomma maculatum* group has consisted of three species: *A. maculatum*, *A. triste*, and *A. tigrinum*. Not only have both *A. parvitarsum* and *A. neumanni* been excluded from the group, they have also been removed from the subgenus *Anastosiella* (Estrada-Peña et al. 2005). Estrada-Peña et al. further proposed removing members of the *Amblyomma ovale* group of ticks from this subgenus, thereby suggesting that *Anastosiella* is comprised entirely of the *A. maculatum* group.

Ecology of *Amblyomma maculatum* group ticks

Amblyomma maculatum sensu stricto (s. s.) is a large, ornate, grassland tick (Figure 1.1) that will parasitize many species of ground-dwelling birds and various mammals, including humans (Goddard 2002, Teel et al. 2010, Portugal and Goddard 2016). Immature stages are rarely collected but are known to parasitize cotton rats (*Sigmodon hispidus*), white-footed mice (*Peromyscus leucopus*), meadowlarks (*Sternella* sp.), and bob-white quail (*Colinus virginianus*), though there are also reports of nymphal *A. maculatum* s. s. recovered from humans (Teel et al. 1998, Goddard 2002, Barker et al. 2004, Goddard 2007, Teel et al. 2010, Moraru et al. 2012, Moraru et al. 2013, Portugal and Goddard 2015). Adult *A. maculatum* s. s. are often found on

larger ungulates and feral swine and are common human biters (Teel et al. 2010). Adults of this tick species are also relatively resistant to desiccation and typically quest in open fields in direct sunlight during the hottest parts of the day (Yoder et al. 2008, Goddard et al. 2011). However, these ticks are often found in areas with high rainfall or near water sources (Paddock and Goddard 2015, Allerdice et al. 2017, Nadolny and Gaff 2017).

Ecological studies of *Amblyomma triste* have shown a strong association of this tick with wetlands (Szabó et al. 2003, Szabó et al. 2007, Venzal et al. 2008). Adult stages of *A. triste* commonly infest marsh deer (*Blastocerus dichotomus*) across South America and are the most common human-biting ticks in Uruguay, although they have also been reported on humans from Argentina, Brazil, and Venezuela (Venzal et al. 2004, Guglielmone et al. 2006). *Amblyomma triste* is also the primary vector for *Rickettsia parkeri* in South America (Venzal et al. 2004, Pacheco et al. 2006, Nava et al. 2008, Conti-Díaz et al. 2009, Romer et al. 2011, Portillo et al. 2013, Monje et al. 2014, Romer et al. 2020).

Like the other two *A. maculatum* group tick species, immature stages of *Amblyomma tigrinum* are commonly found on small rodents and birds; however, adults prefer wild and domestic carnivores (Gonzalez-Acuna et al. 2004, Nava et al. 2006). Adults are occasionally found on humans (Guglielmone et al. 2006), and *A. tigrinum* has recently been identified as a potential vector of *R. parkeri* in South America (Lado et al. 2014, Romer et al. 2014). This tick exhibits a unique ability to colonize various climatic areas, ranging from Chilean lowlands to higher altitudes in the Peruvian Andes (Guglielmone et al. 2003, Gonzalez-Acuna et al. 2004).

Figure 1.1 Life stages of *Amblyomma maculatum*

Comparison of *Amblyomma maculatum* life stages. *Amblyomma maculatum* larva (left), nymph (center), and adult female (right) in relation to size of a penny (USA).

Distribution of *Amblyomma maculatum* group ticks

Amblyomma maculatum group ticks have been reported throughout the Americas. The common name of *A. maculatum* s. s. (Gulf Coast tick) is based on the historical prevalence of this tick along the Gulf Coast region of the United States (Figure 1.2) (Bishopp and Trembley 1945). However, beginning in the middle of the 20th century, the cattle industry facilitated entry and establishment of Gulf Coast ticks into both Kansas and Oklahoma (Teel et al. 2010). Since then, established populations of Gulf Coast ticks have been found as far north as Delaware (Florin et al. 2013, Maestas et al. 2020) and Connecticut (Molaei et al. 2021), and are well established in Virginia (Fornadel et al. 2011), Arkansas (Trout Fryxell et al. 2015), and Kentucky

(Pagac et al. 2014). In fact, the range of this tick has expanded into the Nearctic zone significantly since 1945 (Figure 1.3).

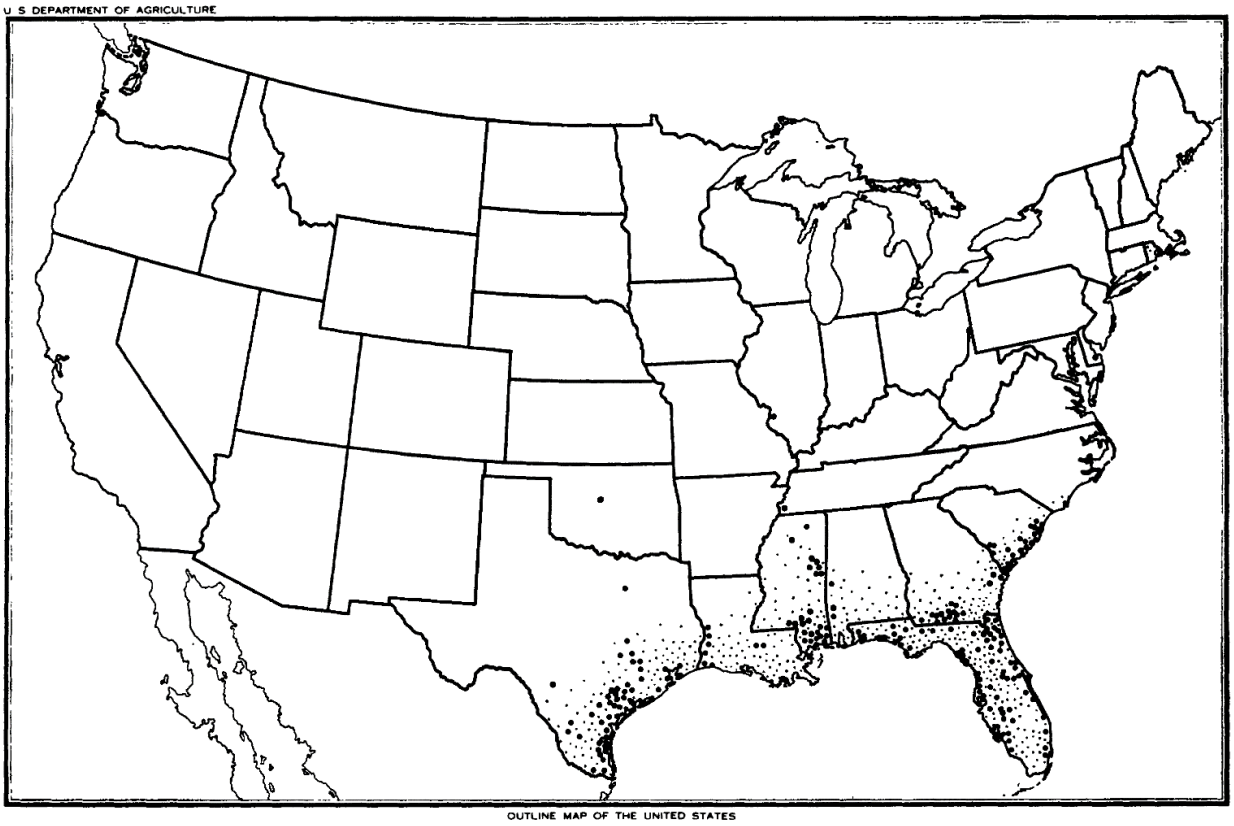

Figure 1.2 Historic distribution of *A. maculatum* in the United States – 1945

From Bishopp and Trembley (1945).

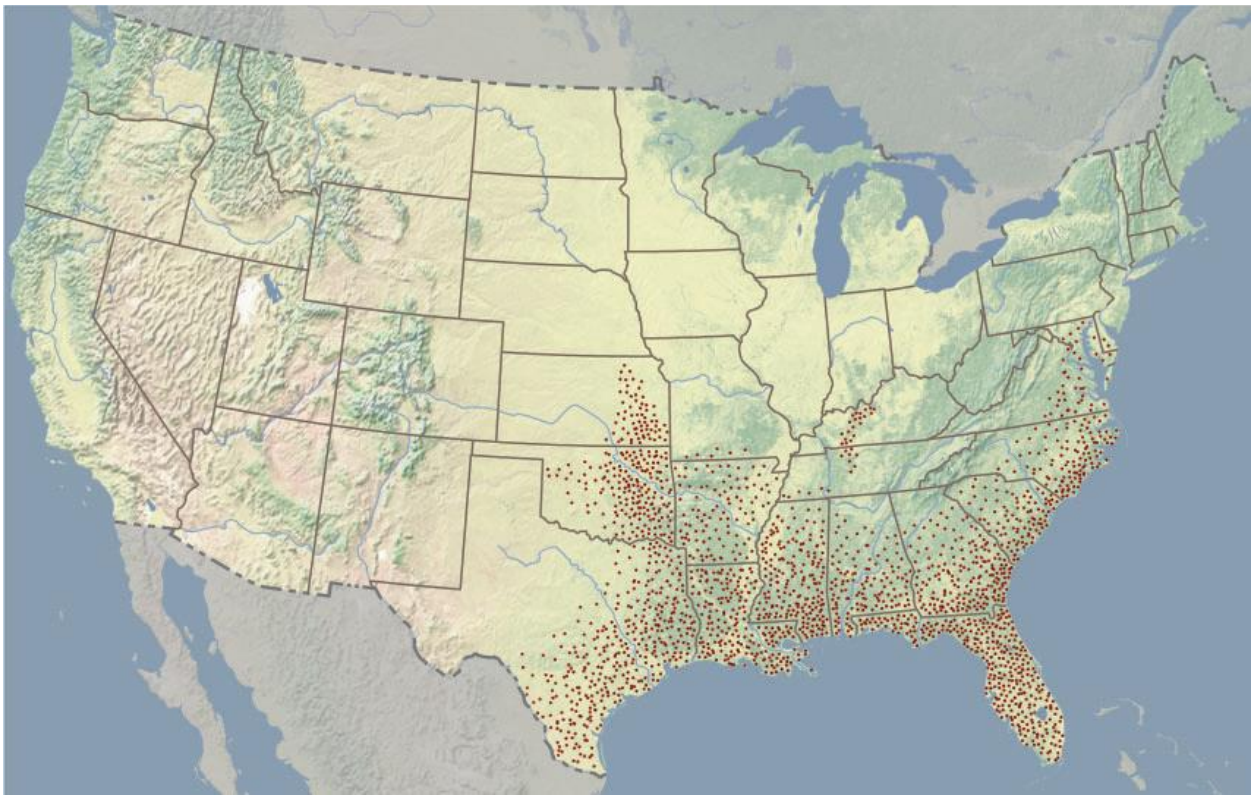

Figure 1.3 Distribution of *A. maculatum* in the United States – 2015

From Paddock and Goddard (2015).

Amblyomma triste was originally described from Uruguay in 1844 and is considered a Neotropical species that spans much of South America (Estrada-Peña et al. 2005). However, new records indicate that this tick exists in North America as well, with collections reported from the states of Sonora and Coahuila in Mexico and what was believed to be *A. triste* from Arizona in the United States (Figure 1.4) (Guzmán-Cornejo et al. 2006, Herrick et al. 2016, Mertins et al. 2010).

Amblyomma tigrinum is widely distributed across the Neotropical realm, spanning South America from Venezuela to Argentina (Figure 1.4) (Guglielmone et al. 2000, Estrada-Peña et al. 2005). Because the adult stage of *A. tigrinum* predominately parasitizes wild and domestic

medium to large carnivores, its distribution is theoretically as unrestricted as that of its hosts, however the known distribution includes only South America.

Figure 1.4 Distribution of *Amblyomma maculatum* group ticks in the Americas. Distribution of *A. maculatum, A. tigrinum,* and *A. triste.* From Estrada-Peña et al. (2005).

The medical and veterinary importance of *Amblyomma maculatum* s. s.

Amblyomma maculatum s. s. has been reported as a major veterinary pest dating back to the early 20th century, when these ticks were designated as the second most economically significant cattle pest behind *Rhipicephalus annulatus* (Say, 1821) (formerly *Boophilus annulatus*) (Bishopp and Hixson 1936). This pest behavior is still relevant in the 21st century, as *A. maculatum* s. s. are notorious for causing a condition in cattle and other ungulates called "gotch ear," an effect of multiple adult *A. maculatum* s. s. feeding in the ears of a host resulting in thickened, edematous, curled ears (Bishopp and Hixson 1936, Edwards 2011). The ticks can cause permanent, severe structural damage to the cartilage of the animals' ears, resulting in swelling and the excretion of a yellow exudate that causes a crust on the ear. This bite damage to cattle has been known to predispose cattle to myiasis caused by the primary screwworm *Cochliomyia homnivorax* (Coquerel, 1858) (previously *Callitroga americana)* (Spicer and Dove 1938), whose females are attracted to oviposit at *A. maculatum* bite sites. While the primary screwworm was eradicated from the United States (Wyss 2000), this medical and veterinary pest still threatens both people and livestock in Central and South America, including in countries where populations of *A. maculatum* group ticks exist.

Additionally, *A. maculatum* s. s. can cause tick paralysis in both humans and livestock (Paffenbarger 1951, Espinoza-Gomez et al. 2011). Tick paralysis is a condition in which an attached tick is associated with ascending paralysis in its host (Rose 1954). This direct effect is thought to be caused by neurotoxins in the tick's saliva, though the exact mechanism is not known (Grattan-Smith et al. 1997). Typically, once the attached tick is removed, paralysis subsides and the mammalian host quickly resumes normal muscle control; however, the

condition can be lethal if the tick is not promptly removed (Gregson 1973, Darrow 1974, Felz et al. 2000, Van Gerpen and Caruso 2005, Remondegui 2012, Diaz 2015).

Amblyomma maculatum s. s. has also been identified as a competent vector in the laboratory for *Ehrlichia ruminantium*, the causative bacterial agent of heartwater in cattle (Uilenberg 1982). Heartwater is a devastating rickettsial disease of various ungulates including domestic cattle that can cause neurological signs as well as an accumulation of pericardial and pleural fluids in affected cattle (Du Plessis et al. 1987). This disease causes significant morbidity and mortality in cattle herds, resulting in huge economic losses. While the disease is not known to be present in North America, the pathogen has been present in the Caribbean over the past fifty years. With the increased trade and movement of animals in the global market, heartwater poses a foreign animal disease threat to the United States, potentially devastating the livestock industry if this pathogen were to be imported.

Amblyomma maculatum s. s. ticks are aggressive human biters and are the primary vectors in the United States of *Rickettsia parkeri*, an emerging human rickettsiosis that causes clinical symptoms similar to, but typically milder than, that of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (Paddock et al. 2008). All stages of *A. maculatum* s. s. will parasitize humans (Goddard 2002, Paddock and Goddard 2015, Portugal and Goddard 2016), and *R. parkeri* infection frequencies in populations of adult Gulf Coast ticks are reported to range from 6% to upwards of 40% in the United States. These ticks are also frequently infected with "*Candidatus* Rickettsia andeanae," a recently discovered rickettsial organism of unknown pathogenicity. Infection rates of "*Ca.* Rickettsia andeanae" range from 0.6% to 10% in the United States, and co-infections of these two rickettsial organisms have been reported in limited numbers (Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al.

2012, Jiang et al. 2012, Nadolny et al. 2014, Pagac et al. 2014, Trout Fryxell et al. 2015, Goddard et al. 2016, Lee et al. 2016, Mays et al. 2016, Allerdice et al. 2017).

Background of Rickettsiales and *Rickettsia parkeri*

The order Rickettsiales consists of obligately intracellular alpha-proteobacteria that typically cycle between vertebrate and invertebrate hosts (Yu and Walker 2006). Within this order is the family Rickettsiaceae, which contains the genus *Rickettsia.* This genus can be further subdivided into four main groups: spotted fever group (SFG), typhus group (TG), transitional group (TRG), and ancestral group (AG) rickettsiae (Gillespie et al. 2007). Transmitted by ticks, fleas, mites, and body lice, many rickettsial bacteria have historically been recognized as human pathogens, including *Rickettsia rickettsii* (SFG, the causative agent of Rocky Mountain spotted fever (RMSF)), *Rickettsia conorii* (SFG, causative agent of Boutonneuse fever), *Rickettsia sibirica* (SFG, causative agent of Siberian tick typhus), *Rickettsia africae* (SFG, causative agent of African tick-bite fever), *Rickettsia prowazekii* (TG, causative agent of epidemic typhus), *Rickettsia typhi* (TG, causative agent of murine typhus), *Rickettsia akari* (TRG, causative agent of mite-borne rickettsialpox), *Rickettsia australis* (TRG, causative agent of Queensland tick typhus), and *Rickettsia felis* (TRG, causative agent of cat-flea rickettsiosis) (Yu and Walker 2006).

All of these rickettsial pathogens cause varying degrees of illness in humans and dogs, with typical symptoms including fever, myalgia, arthralgia, rash, headache, and thrombocytopenia (Socolovschi et al. 2009). Long-term or permanent disabilities from SFG rickettsioses can be more severe and include blindness, deafness, or amputation following gangrene. Fatality rates of SFG rickettsioses are often dependent on the availability of tetracycline class antibiotics and health providers with the knowledge necessary to diagnose

these diseases (Biggs et al. 2016). Thus, fatality rates can be contemporarily as high as 40% in certain parts of Mexico (Alvarez-Hernandez et al. 2017).

Prior to 2002, three *Rickettsia* species were known to cause illness in the United States: *R. akari*, *R. felis*, and *R. rickettsii*. *Rickettsia akari* is transmitted to humans by the house mouse mite, *Liponyssoides sanguineus* (Hirst, 1914) (formerly *Allodermanyssus sanguineus*) and causes a non-fatal rickettsiosis that begins with an eschar at the site of mite attachment followed by a maculopapular rash (Yu and Walker 2006). *Rickettsia felis* also causes a mild rickettsiosis and is found primarily in the cat flea *Ctenocephalides felis* (Bouché, 1835) (Pérez-Osorio et al. 2008), though recent analyses in the United States identified this rickettsial organism in several species of mosquitoes (Barua et al. 2020). However, of the rickettsial pathogens recognized in the U.S. prior to the 21st century, *R. rickettsii* was the only one transmitted by ticks, specifically *Dermacentor variabilis* (Say, 1821) in the eastern United States, *Dermacentor andersoni* Stiles, 1908 in the western United States, and *Rhipicephalus sanguineus* (Latreille, 1806) in some focal regions of the southwestern United States (Jay and Armstrong 2020).

The understanding of tick-borne rickettsial diseases in the United States changed in 2002, however, when a patient presented with a history of tick bites after spending time in grasses near his home in the Tidewater region of southeast Virginia. He exhibited clinical symptoms similar to those of RMSF, including fever, fatigue, headache, myalgia, and generalized rash (Paddock et al. 2004). The case was also notable for the presence of multiple eschars at the sites of tick attachment (Figure 1.5). A skin punch biopsy was performed on one of the eschars and placed into cell culture, resulting in isolation of a rickettsial bacteria from the site, later confirmed as *Rickettsia parkeri* based on sequencing of the portion of an outer membrane protein gene (Paddock et al. 2004). This work conclusively identified *R. parkeri* as a causative agent of

human rickettsiosis, thus confirming this bacterium as the second tick-transmitted rickettsial human pathogen circulating in the United States.

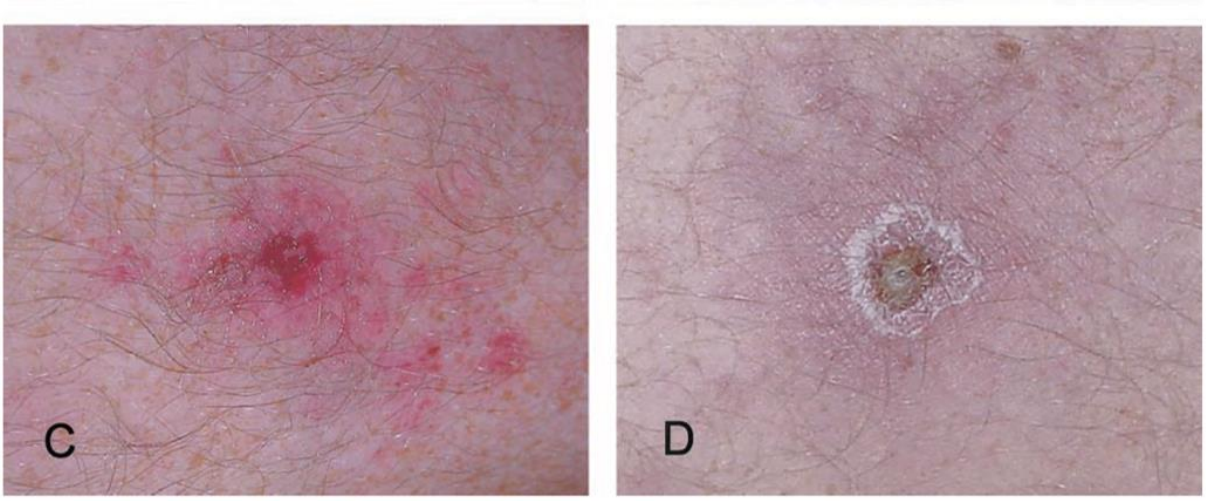

Figure 1.5 Eschars caused by *R. parkeri* rickettsiosis

Eschars from first confirmed human case of Rickettsia parkeri rickettsiosis. From Paddock et al. (2004).

While the first confirmed human case of *R. parkeri* rickettsiosis was published in 2004, the original observation and isolation of *R. parkeri* was decades earlier. In 1939, Ralph R. Parker and colleagues from Rocky Mountain Laboratories isolated a novel *Rickettsia* species from *A. maculatum* that would go on to be formally named "*Rickettsia parkeri*" in 1965 (Parker et al. 1939, Lackman et al. 1965). Even at the time of original isolation, Parker and colleagues surmised that this new *Rickettsia* species could be a cause of human illness based on animal experimentation in guinea pigs; when inoculated with what Parker called "the Maculatum agent," experimental animals developed symptoms of a mild rickettsiosis. Since its confirmation as a human pathogen 65 years later, more than 50 cases of *R. parkeri* have been identified in the United States, (Paddock and Goddard 2015, Herrick et al. 2016, Yaglom et al. 2020). These

cases have typically followed the range of *A. maculatum*, with the furthest west of these cases prior to 2014 located in eastern Texas (Paddock and Goddard 2015).

Epidemiology of *Rickettsia parkeri* and *Amblyomma maculatum* group ticks in southern Arizona

In 2014 two cases of *R. parkeri* rickettsiosis were described in patients who had acquired tick bites while hiking in the Pajarita Wilderness Area in the Pajarito Mountains, a mountain range in southern Arizona that abuts the United States-Mexico border (Herrick et al. 2016). The ticks involved were identified as *Amblyomma triste* based on details of their scutal ornamentation, leg armatures, and festoons (Jones et al. 1972, Guzmán-Cornejo et al. 2011, Martins et al. 2014). While *Amblyomma triste* is a widely distributed Neotropical tick of the *A. maculatum* group found primarily in South America (Guglielmone et al. 2006, Venzal et al. 2008, Nava et al. 2011, Melo et al. 2015), this tick has also been reported from North America, with some historical specimens from Mexico recently identified as *A. triste* (Guzmán-Cornejo et al. 2006, Mertins et al. 2010). Nonetheless, historical reports of *A. triste* in the United States are rare.

Because of the unusual geographical location and arid habitat of the *Amblyomma* ticks identified in southern Arizona, field studies to collect tick specimens were conducted in the region beginning in 2016. These studies were designed to target collection sites within the Madrean Archipelago, a complex of sky islands that extends from northwestern Mexico into the southwestern United States, including Arizona (Warshall 1995). The term “sky island” describes isolated mountain complexes surrounded by lower elevation “seas” that are often comprised of inhospitable terrain (Heald 1951). Southern Arizona contains approximately 30 sky islands of the Madrean Archipelago, including the Pajarito Mountains, which are surrounded by the Sonoran

Desert (Warshall 1995, Moore et al. 2013). A unique ecological feature of sky islands is their arrangement of specific biomes stratified by elevation into several broad zones, ranging from desertscrub at the lowest elevations to mixed conifer forests in the highest elevations (Shreve 1915, Niering and Lowe 1985). In the midrange between these two extremes is the encinal, a unique biome characterized by oak grassland at its lowest elevation and oak woodlands at its upper border (Lowe 1961, Gottfried et al. 1995). Tick collections in Arizona were conducted in sky islands within the encinal.

Resultant ticks from these southern Arizona collections were subsequently morphologically evaluated by a tick taxonomist (Allerdice et al. 2017) and were noted to possess morphological features of both *A. maculatum* and *A. triste*. Specifically, the ventral scutes on the male festoons are more developed than in *A. maculatum* and visible dorsally, and the paired armatures on tibiae II to IV are of unequal thickness similar to that of *A. triste*. In male ticks from Arizona, spiracular plates are also markedly comma shaped as in *A. maculatum* (Allerdice et al. 2017).

Subsequent molecular analyses were performed on *Amblyomma maculatum* group ticks, including the *Amblyomma* ticks from southern Arizona. Results of this study suggest that *Amblyomma maculatum*, *Amblyomma triste*, and the *Amblyomma maculatum* group ticks from southern Arizona are likely one contiguous species across the Americas (Lado et al. 2018). This apparent conspecificity could explain minor morphological differences identified in these ticks; rather than distinguishing separate species, these small differences could constitute local phenotypic adaptations in a single widespread taxon. Because the *Amblyomma* ticks collected in Arizona can only be differentiated based on these small morphological characteristics, the ticks that are responsible for human *R. parkeri* rickettsiosis cases in this state cannot be assigned

conclusively to one species and thus are only known to be members of the *A. maculatum* group of ticks as described by Estrada-Peña et al. (2005). For simplicity, the *Amblyomma maculatum* group ticks from Arizona will heretofore be described as *Amblyomma maculatum* sensu lato (s. l.).

The discovery of established populations of *Amblyomma maculatum* s. l. in desert riparian areas is unexpected; these humidity-loving arthropods appear foreign in this arid environment. The mystery of the ticks' presence could be related to the role of migratory birds in the region. Riparian canyon habitats found in southern Arizona provide a natural migration route for a large diversity of bird species from Central and South America; greater than 100 species of Neotropical birds use these riparian corridors during migrations from Central and South America (Prior-Magee et al. 2007, Brand et al. 2008, Austin et al. 2013), where *A. maculatum* group ticks are endemic (Stotz et al. 1996, Jones et al. 2014, Friggens and Finch 2015). South American migratory birds are often infested with larval and nymphal stages of *A. maculatum* group ticks upon arrival to the United States (Mukherjee et al. 2014, Cohen et al. 2015). In addition, these immature ticks are often infected with *Rickettsia* species, including *R. parkeri* and "*Ca*. R. andeanae" (Mukherjee et al. 2014). It is possible that migratory Neotropical birds regularly transport *Rickettsia*-infected *A. maculatum* group ticks to this region from the southern hemisphere during flights north, thus continuously replenishing tick populations in these corridors. Populations of questing *A. maculatum* group ticks in South America have reported *R. parkeri* infection rates of 3% to 20% in regions of Uruguay and Argentina (Pacheco et al. 2006, Nava et al. 2008, Cicuttin and Nava 2013, Monje et al. 2014, Melo et al. 2015, Monje et al. 2016), suggesting that *R. parkeri* may be transported with these ticks as well.

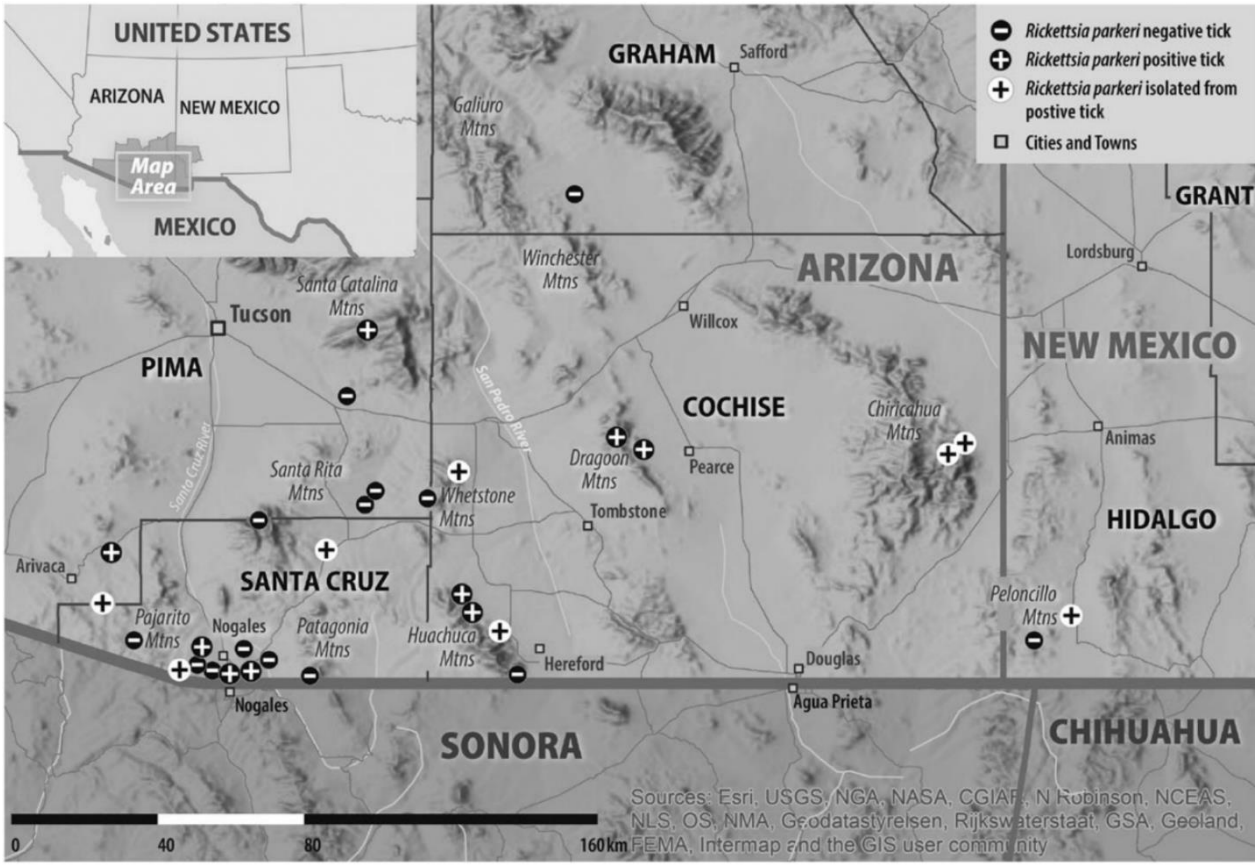

Figure 1.6 Map of tick collection sites in the southwestern United States

Map of southern Arizona and New Mexico identifying collection sites for *Amblyomma maculatum* senso lato as well as *Rickettsia parkeri*. From Hecht et al. (2020).

Field investigations performed in southern Arizona, New Mexico, and western Texas during 2016 - 2019 revealed several populations of *R. parkeri*-infected *A. maculatum* s. l. (Figure 1.6), with infection frequencies ranging from 21% to 40% (Allerdice et al. 2017, Hecht et al. 2020, Paddock et al. 2020), similar to rates described previously in *A. maculatum* group ticks collected from the southeastern and mid-Atlantic United States. In Arizona, populations of *A. maculatum* s. l. were identified in Santa Cruz county near the site of the first confirmed human cases from this state (Herrick et al. 2016, Allerdice et al. 2017). Additional populations were also found in the neighboring counties of Pima and Cochise (Allerdice et al. 2017, Hecht et al. 2020).

While no human cases of *R. parkeri* rickettsiosis have yet been reported from New Mexico, an investigation in Hidalgo County yielded *R. parkeri*-positive *A. maculatum* s. l. (Figure 1.6) (Hecht et al. 2020). In Texas, field studies identified *R. parkeri*-positive *A. maculatum* s. l. tick populations in both Jeff Davis and Brewster counties (Paddock et al. 2020).

The discovery of these tick populations in the southwestern United States is consonant with historical reports that date back to 1942 describing individual specimens of *A. maculatum* group ticks collected from humans, dogs, and cattle from southern Arizona (Cooley and Kohls 1944, Mertins et al. 2010). However, the question of these ticks' true species identity remains unanswered. Nevertheless, whatever the actual species of these ticks may be, the high level of rickettsial infections detected in these desert tick populations (coupled with the proclivity of *A. maculatum* group ticks to parasitize humans) suggests a significant public health risk for those spending time in the American Southwest.

Purpose of the study

The taxonomy of *Amblyomma maculatum* group ticks has been dynamic throughout its history. The recent discovery of members of this tick group in unexpected habitats in southern Arizona and other areas of the southwestern United States and northern Mexico highlights the need for more complex analysis of both the tick group and the rickettsial pathogen it is known to transmit. Little work has been done to clarify potential genetic variation of strains of *R. parkeri*, a human pathogen transmitted by multiple vectors across two continents. To understand the true public health risk posed by *R. parkeri* in areas endemic for *A. maculatum* group ticks, the pathogen itself needs to be better understood. In parallel, understanding this public health risk requires a deeper knowledge of the systematics of *A. maculatum* group ticks, especially in southern Arizona, where these ticks are transmitting *R. parkeri*. These tick populations' presence

in surprising environments challenges the known and expected range of these species in the United States. Identifying the potential origin of these tick populations and their relationships to other *A. maculatum* group ticks could provide important context to how these ticks are able to colonize this unexpected habitat. Therefore, the purpose of this study is to help clarify the systematics and taxonomic status of the *A. maculatum* group ticks and *R. parkeri* in southern Arizona.

Dissertation Objectives

1. Under laboratory conditions, determine reproductive compatibility between *Amblyomma maculatum* s. l. from Arizona and *A. maculatum* sensu stricto (s. s.) from Georgia by performing a cross-mating study.
2. Develop and optimize polymorphic microsatellite loci for use in future analysis of *Amblyomma maculatum* group ticks.
3. Characterize genetic divergence within North American strains of *R. parkeri* s. s. and between North and South American strains of *R. parkeri* s. l. through the development and application of a multi-locus sequence typing analysis of *R. parkeri* isolates and *R. parkeri*-positive tick lysates.

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CHAPTER II

REPRODUCTIVE INCOMPATIBILITY BETWEEN *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE) GROUP TICKS FROM TWO DISJUNCT GEOGRAPHICAL REGIONS WITHIN THE UNITED STATES

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Abstract

The *Amblyomma maculatum* Koch group of ixodid ticks consists of three species: *A. maculatum*, *A. triste*, and *A. tigrinum*. However, since Koch described this group in 1844, the systematics of its members has been the subject of ongoing debate. This is especially true of *A. maculatum* and *A. triste*; recent molecular analyses reveal insufficient genetic divergence to separate these as distinct species. Further confounding this issue is the discovery in 2014 of *A. maculatum* group ticks in southern Arizona, USA that share morphological characteristics with both *A. triste* and *A. maculatum*. To biologically evaluate the identity of *Amblyomma maculatum* group ticks from southern Arizona, we analyzed the reproductive compatibility between specimens of *A. maculatum* group ticks collected from Georgia and southern Arizona, USA. Female ticks from both Arizona and Georgia were mated with males from both the Georgia and Arizona *Amblyomma* populations, creating 2 homologous and 2 heterologous F1 cohorts of ticks:

GA ♀/GA ♂, AZ ♀/AZ ♂, GA ♀/AZ ♂, and AZ ♀/GA ♂. Each cohort was maintained separately into the F2 generation with F1 females mating only with F1 males from their same cohort. Survival and fecundity parameters were measured for all developmental stages. The observed survival parameters for heterologous cohorts were comparable to those of the homologous cohorts through the F1 generation. However, the F1 heterologous females produced F2 egg clutches that did not hatch, thus indicating that the Arizona and Georgia populations of *A. maculatum* group ticks tested here represent different biological species.

Introduction

Taxonomic relationships among the *Amblyomma maculatum* tick group have been contested and dynamic for more than 175 years since Koch initially described several species belonging to this group (Koch 1844, Kohls 1956). Former members include *Amblyomma rubripes* Koch, 1844, *Amblyomma ovatum* Koch, 1844, *Amblyomma parvitarsum* Neumann, 1901, *Amblyomma neumanni* Ribaga, 1902, and multiple other taxa which have since been collapsed in synonymy (Kohls 1956, Camicas et al. 1998). The most recent analysis defines only three valid species: *A. maculatum* Koch, 1844, *Ambylomma triste* Koch, 1844, and *Amblyomma tigrinum* Koch, 1844 (Estrada-Peña et al. 2005). While *A. tigrinum* is morphologically distinct, *A. triste* and *A. maculatum* are extremely difficult to separate morphologically and are distinguished by relatively few diagnostic features, though geographical data has historically proven useful in separating these species due to their largely allopatric distributions (Estrada-Peña et al. 2005, Mertins et al. 2010).

Amblyomma maculatum are aggressive human biters and are primary vectors in the United States of *Rickettsia parkeri*, the causative agent of an emerging rickettsiosis that causes a

disease of humans similar to but typically milder than that caused by *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF) (Paddock et al. 2008, Teel et al. 2010, Paddock and Goddard 2015). To date, no known fatalities have been reported from infection with *R. parkeri*. All stages of *A. maculatum* will parasitize humans (Goddard 2002, Paddock and Goddard 2015, Portugal and Goddard 2016), and *R. parkeri* infects adult *A. maculatum* at frequencies that generally range from 20% to 40% in the United States (Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Nadolny et al. 2014, Pagac et al. 2014, Trout Fryxell et al. 2015, Lee et al. 2016, Mays et al. 2016, Allerdice et al. 2017).

Amblyomma triste are Neotropical ticks most commonly reported from Argentina, Brazil, Uruguay, and Venezuela (Guglielmone et al. 2006, Venzal et al. 2008, Nava et al. 2011, Melo et al. 2015) and are considered important vectors of *R. parkeri* in Argentina and Uruguay (Venzal et al. 2004, Silveira et al. 2007, Nava et al. 2008, Conti-Díaz et al. 2009, Romer et al. 2011, Portillo et al. 2013, Melo et al. 2015). Until recently, the northernmost collections of *A. triste* were reported from the Mexican states of Coahuila and Sonora (Guzmán-Cornejo et al. 2006). In 2010, however, Mertins et al. reported the recovery of an *A. triste* tick from cattle entering the United States from Mexico. A subsequent examination of archival tick specimens identified as *A. maculatum* and submitted to the United States Department of Agriculture (USDA) as part of the National Tick Surveillance Program revealed 15 specimens with morphological features of *A. triste.* An additional 12 new records of *A. triste* were submitted from several counties in Arizona, Texas, and Coahuila between 2004 – 2009 (Mertins et al. 2010).

In 2016, two cases of *R. parkeri* rickettsiosis were described in patients who had acquired tick bites in a mountainous region in southern Arizona that abuts the United States-Mexico border (Herrick et al. 2016). Ticks identified as *A. triste* based on their scutal ornamentation, leg armatures, and festoons (Jones 1972, Mertins et al. 2010, Guzmán-Cornejo et al. 2011, Martins et al. 2014) were associated with both cases. Subsequent field surveys identified *R. parkeri*-infected *Amblyomma* ticks from this region and other proximate sites in southern Arizona, New Mexico (Allerdice et al. 2017, Hecht et al. 2020), and western Texas (Paddock et al. 2020). These specimens were noted to possess morphological features of both *A. maculatum* and *A. triste*, precluding a definitive morphological assignment to either species (Allerdice et al. 2017, Lado et al. 2018). A recent evaluation of mitochondrial and nuclear gene sequences of these and other members of the *A. maculatum* group suggests that *A. triste* and *A. maculatum* are not sufficiently divergent to separate as species (Lado et al. 2018). This study by Lado et al. further identified 4 morphotypes comprising specimens defined as *A. maculatum* or *A. triste*, based on subtle morphological differences among populations from North, Central and South America. Based on this analysis, *Amblyomma* ticks from southern Arizona represent a unique morphotype, distinct from type specimens of *A. maculatum* sensu stricto (s. s.) and *A. triste* s. s.

To further characterize the identity of the *Amblyomma maculatum* group ticks in the southwestern United States, we performed a crossbreeding experiment to evaluate the reproductive compatibility of these ticks with *A. maculatum* s. s, to better determine if these represent the same or separate biological species.

Materials and Methods

Tick collection

Questing adult *A. maculatum* s. s. were collected from Panola Mountain State Park in Rockdale County, Georgia, USA on 5 July and again during 18-19 July 2017 (Figure 2.1a). Questing adult *A. maculatum* group ticks (heretofore designated as *A. maculatum* sensu lato (s. l.)) were collected from San Pedro Riparian National Conservation Area in Cochise County, Arizona, USA on 14 July 2017 (Figure 2.1b). Ticks were collected as questing specimens from vegetation using flannel cloth flags and were transported live to CDC, where they were identified morphologically using standard taxonomic keys (Estrada-Peña et al. 2005). Specimens were rinsed with a solution of water and liquid dish soap and separated by sex into individual 12 x 75 mm polystyrene tubes in groups of 10 as previously described (Levin and Schumacher 2016).

Figure 2.1 Tick collecting

Collections of *Amblyomma maculatum* sensu stricto from Panola Mountain State Park in Georgia (1a) and *Amblyomma maculatum* sensu lato from San Pedro Riparian National Conservation Area in Arizona (1b).

Crossbreeding assessments

All animal studies were performed according to the protocol 2904LEVRABC approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention and the previously published manual for maintenance of ixodid ticks (Levin and Schumacher 2016). Throughout the study, individual naïve 4-month-old female New Zealand white rabbits (*Oryctolagus cuniculus*) were utilized to feed each cross. Rabbits were infested at the same time and were housed in separate cages in the same room to minimize potential environmental variances. Infested rabbits were checked daily for dead, detached, or replete ticks.

There was no co-feeding of different crosses on the rabbits, and experimental rabbits were humanely euthanized after the completion of each feeding.

For the initial F0 adult cross, groups of 24 unfed virgin *Amblyomma* females from Georgia or Arizona were placed in individual stockinette feeding bags on the shaved dorsa of the rabbits along with 24 males from either Georgia or Arizona, with one bag per rabbit (Figure 2.2). This created two homologous (GA ♀/GA ♂ and AZ ♀/AZ ♂) and two heterologous (GA ♀/AZ ♂ and AZ ♀/ GA ♂) breeding crosses. This parental F0 generation of ticks was allowed to feed to repletion, at which point detached replete females were weighed individually, and the duration to engorgement was recorded for each female. Attached males were removed with forceps on the day the last replete female detached. Detached males and all previously collected dead ticks were cleaned with water and liquid dish soap to dislodge any external contaminants and isolated in individual cryovials for morphological analysis.

Replete gravid females (Figure 2.3) were placed in individual 11.1 ml polystyrene containers and maintained in a high humidity incubator (Levin and Schumacher 2016) under identical conditions of 90 % humidity, 22 ℃, and a 16/8 light/dark photoperiod. Females were monitored daily to assess for oviposition. The pre-oviposition period was determined by calculating the number of days between the day replete females detached and the day oviposition started. Eggs were removed daily from ovipositing females and placed into a separate 11.1 ml polystyrene tube which was maintained in the same incubator with the laying females. At the end of oviposition, egg mass weight corresponding to each female was recorded. To quantify the ability of each replete female to convert its bloodmeal to eggs, a bloodmeal conversion index (BMCI), was calculated by dividing weight of the complete egg mass by engorgement weight of

the corresponding replete female. Evidence of larval eclosion for each egg clutch was monitored daily, and an incubation period was calculated as the difference between date the female began oviposition and date the first larvae were detected in the clutch. Hatching success was estimated as previously described (Drummond et al. 1973) and recorded as successful for those egg clutches that exhibited over 90% eclosion.

Figure 2.2 Preparation of experimental rabbits

Preparation of anesthetized experimental New Zealand white rabbits (*Oryctolagus cuniculus*). The left panel demonstrates shaving the dorsum of the rabbit and applying a hobble to the hind legs. In the panel on the right, the hobble is complete, and the dorsum is nearly shaved.

Figure 2.3 Replete *A. maculatum* s. s. female ticks

For the larval feedings, four entire F1 larval cohorts from each of the F0 parental crosses were placed in separate labeled stockinette bags on naïve rabbits. Larval cohorts were selected for placement based on highest observed percentage of hatching. Larvae were fed in parallel on separate rabbits in individual cages in the same room under identical conditions, as described previously. Replete and dead larvae were removed daily using a vacuum pump system (Figure 2.4) (Levin and Schumacher 2016). The first 2000 replete larvae in each cohort to detach were cleaned with water and liquid dish soap and separated into groups of 100 specimens into each of twenty 11.1 ml polystyrene tubes. Larvae were monitored daily and molting success for each larval cohort was recorded. If mold appeared at any point during the molt, the affected tube was removed and placed in a separate incubator under the same conditions as the non-moldy larvae. Moldy larvae were subsequently excluded from analyses of the molting success.

Molted F1 nymphs were allowed to feed on rabbits as described previously. Cohorts of 350 F1 nymphs each were placed in three separate bags on naïve rabbits, comprising a total of 1,050 nymphs per rabbit. Nymphs from each cross were fed in parallel on separate rabbits in individual cages in the same room under identical conditions. Replete nymphs were removed daily by vacuum pump. The replete nymphs were cleaned with water and liquid dish soap and separated into groups of 10 in separate 12 x 75 mm polystyrene tubes. Feeding success was evaluated as the percentage of nymphs that fed to repletion in each bag. Replete nymphs were monitored daily for eclosion, and molting success for each nymphal cohort was recorded.

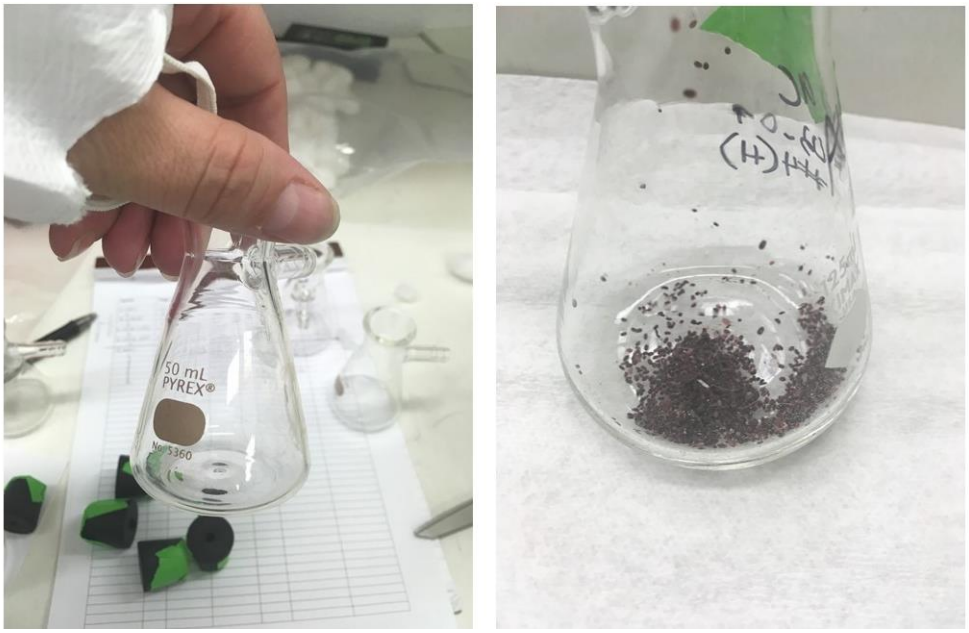

Figure 2.4 Vacuum apparatus for tick collection

Components of the vacuum system for collection of larvae from rabbits. The left panel shows an empty Erlenmeyer flask and black stoppers. The stoppers go in the top of the flask, which is then attached to a vacuum hose through the projection on the side of the flask. The right panel shows a flask that contains live and dead *Amblyomma maculatum* larvae collected from a rabbit for this experiment.

Twenty-four resultant F1 females from each cross were placed on a separate naïve rabbit paired with 24 males from the same cross to assess their fertility and fecundity. Ticks were placed at approximately 24 days post-molt. Males and females were selected from separate genetic lineages within crosses to limit potential inbreeding. Females were allowed to feed to repletion, and survival parameters of feeding duration, engorgement weight, BMCI, preoviposition period, and hatching success of the F2 eggs were measured as described above. Males were removed after females had fully engorged and were subsequently cleaned with water and liquid dish soap and placed into individual cryovials for morphological analysis.

Morphology

Morphological analysis of the experimental ticks was performed qualitatively. Gross morphologies of the adult F0 parental ticks as well as the adult F1 homologous cohorts and hybrid ticks were analyzed using a Zeiss Stemi 305 dissecting microscope and compared to the four morphotypes presented in Lado et al. 2018. Prominent characters were assessed against descriptions provided by Lado et al., with a primary focus on the tibial armatures on legs II-IV of both male and female specimens as well as the spiracular plates in male ticks.

Statistical Analyses

Linear regression was used to evaluate differences among crosses for the continuous fecundity variables: female feeding duration, engorgement weight, preoviposition period, egg mass, and minimum incubation period to eclosion. Crosses were compared using differences of mean (95% CIs) estimated from the regression fits and utilizing sandwich estimators of variance in multiple comparisons to account for heteroscedasticity (Bretz et al. 2011).

Logistic regression was used to evaluate differences among crosses for binary fecundity variables: female feeding success, percentage of replete females ovipositing, hatching success, and female-to-male ratio. Models were fit using the Firth correction, and sandwich estimators were used to account for heteroscedasticity in multiple comparisons of differences (95% CIs) between log odds.

To evaluate differences in blood meal conversion indices (BMCI), which range 0 to 1, an arcsine transformation was used on the outcome BMCI before employing linear regression to evaluate differences among crosses.

Data were also collected on larvae and nymphs: larval molting success, nymphal feeding success, and nymphal molting success. Logistic regression fit using the Firth correction was used to evaluate differences among F1 generation crosses, and sandwich estimators of variance were used in multiple comparisons to account for heteroscedasticity among F1 crosses.

Results

F0 → F1 Generations

The F0 cohorts fed successfully on their respective rabbits. For both homologous cohorts, 23/24 female ticks fed to repletion; one female from the Georgia homologous cohort only partially fed before being removed on day 15 post infestation and one female from the Arizona homologous cohort died four days post infestation. Biological parameters for these four cohorts were very similar, though females from AZ completed their repletion approximately two days faster than those from GA, regardless if they mated with homologous or heterologous males F0

Table 2.2 Summary of fertility and fecundity parameters for the adult F0 and F1 ticks.

	F0 → F1				**F1 → F2**			
	Homologous Cohorts		**Heterologous Cohorts**		**Homologous Cohorts**		**Heterologous Cohorts**	
	GA ♀ / GA ♂ n = 24	**AZ ♀ / AZ ♂ n = 24**	**GA ♀ / AZ ♂ n = 24**	**AZ ♀ / GA ♂ n = 24**	**GA ♀ / GA ♂ n = 20**	**AZ ♀ / AZ ♂ n = 20**	**GA ♀ / AZ ♂ n = 20**	**AZ ♀ / GA ♂ n = 20**
Female feeding success (%)***	95.8 (79.8, 99.8)[a,b]	95.8 (79.8, 99.8)[a,b]	95.8 (79.8, 99.8)[a,b]	100 (86.2, 100)[a]	100 (83.9, 100)[a]	100 (83.9, 100)[a]	10.0 (2.8, 30.1)[c]	70.0 (48.1, 85.5)[b]
	n = 23	**n = 23**	**n = 23**	**n = 24**	**n = 20**	**n = 19**	**n = 2**	**n = 14**
Female feeding duration (days)**	10.5 (10.1, 10.9)[a]	8.17 (7.77, 8.58)[b]	10.6 (10.2, 11.0)[a]	8.29 (7.76, 8.83)[b]	10.4, (9.89, 10.9)[a]	10.1 (9.51, 10.7)[a]	12.5 (6.15, 18.9)*	12.1 (11.2, 13.1)[c]
Engorgement weight (g)**	0.93 (0.84, 1.03)[a]	0.87 (0.81, 0.93)[a]	0.94 (0.82, 1.05)[a]	0.84 (0.74, 0.94)[a]	0.99 (0.87, 1.11)[a]	0.87 (0.79, 0.95)[a]	0.32 (0.25, 0.38)*	0.94 (0.74, 1.14)[a]
Replete females ovipositing (%)***	100 (85.7, 100)[a]	100 (85.7, 100)[a]	100 (85.7, 100)[a]	100 (86.2, 100)[a]	100 (83.9, 100)[a]	95.0 (76.4, 99.7)[a]	100 (34.2, 100)*	100 (78.5, 100)[a]
Pre-oviposition period (days)**	5.21 (4.48, 5.60)[a]	4.96 (4.58, 5.34)[a]	7.05 (5.99, 8.10)[b]	4.62 (4.26, 4.99)[a]	6.75 (6.45, 7.05)[b]	5.21 (4.87, 5.55)[a]	5.5 (0, 11.9)*	5.43 (4.84, 6.02)[a]
Egg mass weight (g)**	0.55 (0.50, 0.61)[a]	0.51 (0.47, 0.55)[a]	0.54 (0.45, 0.63)[a,b]	0.50 (0.43, 0.57)[a]	0.57 (0.47, 0.66)[a,b]	0.55 (0.48, 0.63)[a,b]	------	0.71 (0.61, 0.82)[b]
Blood meal conversion index **	57.5 (55.0, 60.0)[a]	58.0 (55.7, 60.3)[a]	55.7 (51.2, 60.2)[a]	58.7 (54.7, 62.8)[a]	56.7 (52.6, 60.9)[a]	63.7 (59.6, 67.9)[a]	------	60.8 (54.9 66.6)[a]
Minimum incubation period to eclosion (days) **	36.0, (35.1, 36.9)[a,b]	36.0 (35.3, 36.7)[a,b]	34.3 (33.4, 35.3)[a]	36.5 (35.9, 37.1)[b]	33.3 (32.8, 34.3)[a]	34.6 (33.6, 35.5)[a,b]	------	------
Hatching success (%)***	100 (85.7, 100.0)[a]	100 (85.7, 100.0)[a]	100 (85.1, 100.0)[a]	100 (86.2, 100.0)[a]	100 (83.9, 100.0)[a]	100 (83.9, 100.0)[a]	0 (0, 65.8)*	0 (0, 21.5)[b]

All cells show means with 95% confidence intervals for each cohort. Superscripts that differ indicate significant statistical difference between crosses.

* This generation-cross group was not included in pairwise comparison analysis due to small sample size.

** Linear regression and t tests were used to test differences in means between cohorts.

*** Logistic regression and t tests were used to test differences in log-odds between cohorts.

(Table 2.1). There was no statistically significant difference in duration to engorgement among cohorts based on the origin of the female and irrespective of the origin of the male, or among engorgement weight of the F0 females across the four cohorts (range 0.84 – 0.94 g). Similarly, there were no statistically significant differences in weights among egg clutches from F0 females, which ranged from 0.50 g – 0.55 g. Blood meal conversion indices for these ticks ranged from 55.7 – 58.7 % and were not statistically significantly different for any of the cohorts. The GA ♀/AZ ♂ egg clutches hatched approximately two days more quickly (34.3 days) than the reciprocal cross (36.5 days), which did not vary statistically significantly from the homologous cohorts (Table 2.1).

Table 2.3 Data for F1 nymphs and larvae

	Homologous Cohorts		**Heterologous Cohorts**	
	GA ♀ / GA ♂	**AZ ♀ / AZ ♂**	**GA ♀ / AZ ♂**	**AZ ♀ / GA ♂**
	n = 2765	**n = 4997**	**n = 1410**	**n = 3700**
Larvae molting success (%)	93.5 (92.5, 94.4)[a]	97.3 (96.8, 97.7)[b]	93.7 (92.3, 94.8)[a,b]	98.0 (97.4, 98.4)[a,b]
	n = 1050	**n = 1050**	**n = 1050**	**n = 1050**
Nymph feeding success (%)	56.3 (53.3, 59.3)[a]	88.7 (86.6, 90.4)[b]	83.7 (81.4, 85.8)[b]	87.7 (85.6, 89.6)[b]
Nymph molting success (%)	94.9 (92.8, 96.4)[b]	98.0 (96.9, 98.7)[a,b]	95.4 (93.9, 96.6)[a,b]	99.0 (98.2, 99.5)[a]
Female-to-male ratio	1.20 (1.02, 1.42)[a,b]	1.19 (1.04, 1.36)[a]	1.20 (1.05, 1.38)[a,b]	1.71 (1.50, 1.96)[b]

Summary of the F1 nymphs and larvae, including nymphal feeding success, larval and nymphal molting success, and female-to-male ratio. Superscripts that differ indicate significant statistical difference between crosses.

Molting success of fed F1 larvae from all four cohorts was 93.5 – 98.0% (Table 2.2). Molting successes of the heterologous cohorts did not differ from each other or from the homologous cohorts, however the two homologous cohorts showed a statistically significant difference in molting, with a greater degree of molting success in the AZ ♀/AZ ♂ cohort (Table

2.2). Nymphal feeding success was similar for three of the four F1 cohorts, however the nymphs from the Georgia F1 homologous cohort fed at a much lower success rate (56.3%) than the other three (83.7 – 88.7%). The molting success of F1 nymphs was greater than 95% (range 95.4 – 99%) for all but the F1 Georgia homologous cohort (94.9%), and the ratio of females to males ranged from 1.19 in the Arizona F1 homologous cohort to 1.71 in the F1 AZ ♀/GA ♂ cohort (Table 2.2).

F1 → F2 Generations

The first attempt to cross the F1 adult hybrids in February 2018 ended early due to two experimental rabbits developing a clostridial infection unrelated to the crossbreeding study. Data collected in this feeding were subsequently excluded from statistical analyses in Table 2.1. The rabbits infested with the AZ ♀/AZ ♂ F1 homologous cohort and the GA ♀/AZ ♂ F1 hybrid cohort developed clostridial infections and were euthanized nine days after tick placement. At the time of euthanasia, nine replete females had been collected from the AZ ♀/AZ ♂ F1 homologous cohort and no replete females had been collected from the GA ♀/AZ ♂ F1 hybrid cohort. These ticks as well as all partially engorged ticks removed from these two rabbits were cleaned and separated into individual 11.1 ml polystyrene tubes to allow for oviposition. All nine of the fully engorged ticks from the AZ ♀/AZ ♂ F1 homologous cohort laid eggs, as did 13 of the 16 partially engorged females removed from the euthanized rabbit. All of these egg clutches hatched at a rate of >90%. Eight of the partially engorged ticks from the GA ♀/AZ ♂ F1 hybrid cohort laid a small clutch of eggs, however none of the eggs from this cohort hatched.

The remaining two healthy rabbits infested with the GA ♀/GA ♂ F1 homologous cohort and the AZ ♀/GA ♂ F1 hybrid cohort continued in the experiment. Seven of the ticks from the GA ♀/GA ♂ F1 homologous cohort came off the rabbit into the cage and were damaged when

the rabbit broke its hobble and scratched the bag open eight days into the experiment. None of these seven ticks laid eggs. The remaining 17 females of the GA ♀/GA ♂ homologous cohort fed to repletion and laid egg clutches, all of which hatched at a rate of >90%. Twenty of the 24 AZ ♀/GA ♂ F1 hybrid cohort females fed to repletion. The remaining four female ticks were removed on day 17 post infestation. All 20 of the replete hybrid females laid eggs. No hatched larvae were detected until day 52 post oviposition, when 4 individual F2 larvae were detected in a single egg batch. These 4 hybrid larvae were sluggish and died within 24 hours after hatching (Figure 2.5).

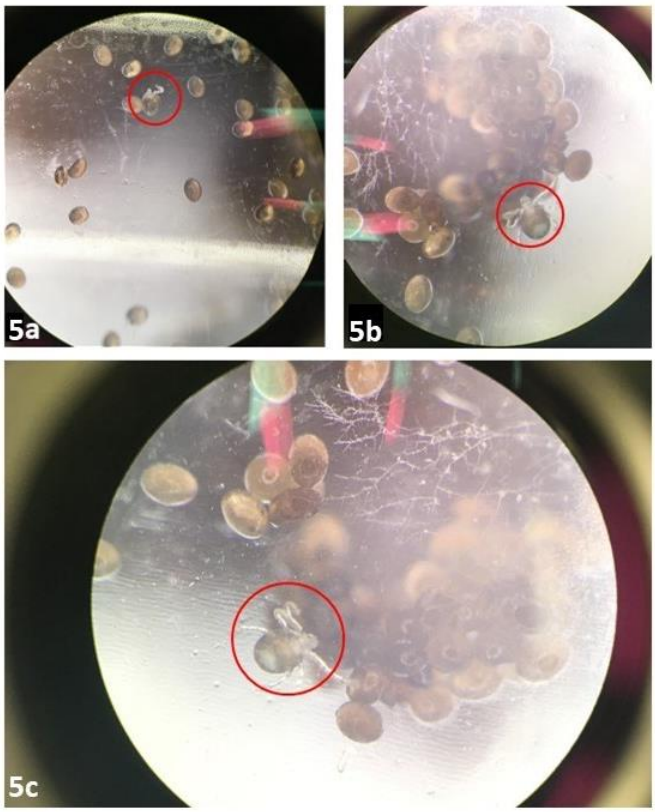

Figure 2.5 F2 AZ ♀/GA ♂ larvae

F2 AZ ♀/GA ♂ larvae circled in red. Panel 5a shows a larva that has not completely hatched. Panels 5b and 5c show larvae that hatched and died shortly after. Note the presence of mold.

The F1 adult feeding was repeated in late March 2018 with 20 pairs of ticks per rabbit as opposed to the 24 pairs that were fed in February. Only data collected during this second attempt were used to calculate the statistics in Table 2.1. Additionally, F1 GA ♀ /AZ ♂ ticks were excluded from all generation-cross group analyses with the exception of adult female feeding success. The low sample size of this generation-cross group following feeding lacks sufficient information to yield reliable results.

All females from the GA ♀/GA ♂ F1 homologous cohort fed to repletion within an average of 10.4 days and laid egg clutches which hatched at a rate of >90%. Females from the

AZ ♀/AZ ♂ F1 homologous cohort all fed to repletion within an average of 10.1 days, and 19/20 laid egg clutches which hatched at a rate of >90%. The single female in this cohort that did not oviposit was damaged during cleaning and then discarded. There was no statistically significant difference between feeding duration of the F1 homologous cohorts as was seen in the F0 feeding; the F1 AZ ♀/AZ ♂ homologous cohort fed similarly to both the F0 and F1 GA ♀/GA ♂ homologous cohorts (Table 2.1). Egg clutches for the Georgia and Arizona F1 homologous cohorts weighed an average of 0.57 and 0.55 g respectively and were not statistically significantly different from any of the other F0 or F1 cohorts.

The GA ♀/AZ ♂ F1 hybrid cohort began engorging at approximately the same time as the homologous cohorts, however most of the ticks stopped engorging by day 7 post infestation. The cuticle of attached partially engorged ticks appeared dry and leathery (Figure 2.6). All attached females on the rabbit were paired with a male, however only two replete females were collected: one on day 12 post infestation and one on day 13. On day 16 post infestation, the rabbit housing these ticks was humanely euthanized and 18 partially engorged ticks were removed. The two replete females and 6/18 partially engorged females from this cohort laid egg clutches. However, none of these F2 hybrid eggs hatched (Table 2.1).

Figure 2.6 Ticks feeding

Images of F1 hybrid ticks feeding. Panel 6a shows AZ ♀/GA ♂, which fed relatively successfully. The ticks in panel 6b are from the GA ♀/AZ ♂ hybrid cohort. Note the dry, leathery appearance of the cuticle for the ticks in panel 6b.

Adult ticks from the AZ ♀/GA ♂ F1 hybrid cohort appeared to feed better than the reciprocal hybrid cohort, with 14/20 replete females detaching within two weeks post infestation; however, this cohort fed to repletion at an average of 12.1 days, a statistically significant difference of approximately two days longer when compared to the homologous cohorts. The remaining six partially engorged females were removed from this rabbit when it was euthanized on day 16 post infestation. All 14 of the replete females and 3/6 of the partially engorged females from this cohort laid egg clutches. However, none of these F2 hybrid eggs hatched (Table 2.1).

A final attempt was made in June 2018 to determine if the GA ♀/AZ ♂ F1 hybrid cohort would engorge. Ten female and 10 male F1 hybrid ticks were placed on a naïve rabbit. Because this was an attempt to qualitatively verify whether or not these ticks would engorge, there was no

homologous control group fed at the same time. The ticks were visually inspected once attached to ensure that the females were paired with males. After 14 days, no females had fed to repletion, and all of the attached partially engorged females appeared pale and leathery. The rabbit was humanely euthanized on day 15 post infestation, and the partially engorged ticks were removed and saved for morphological analysis.

Morphological evaluation

All homologous adults from the F0 generation (24 ♀ and 24 ♂ from each cross) and 80 from each F1 cohort (40 ♀ and 40 ♂ from each cross) were examined using a dissecting microscope and compared to morphotypes described in Lado et al. (Lado et al. 2018). The F0 adults were analyzed post feeding. Descriptions of the morphotypes for each homologous cohort generally matched the respective observed morphology of the ticks. The tibial armatures on legs II-IV of the ticks from Arizona were consistently of unequal thickness. The Arizona male spiracular plates were consistently comma shaped, and female specimens all contained a central brown spot that reached the posterior margin of the scutum (Figure 2.7, d – f). The *A. maculatum* s. s. ticks from Georgia were uniformly consistent with classic descriptions of this species, with tibial armatures on legs II-IV of equal thickness, comma shaped spiracular plates in males, and central brown spots that often do not reach the posterior margin of the scutum (Figure 2.7, a – c) (Kohls 1956, Camicas et al. 1998, Estrada-Peña et al. 2005, Lado et al. 2018).

For the hybrid cohorts, examination of 80 F1 adult hybrid ticks (40 ♀ and 40 ♂) from each cross revealed a mix of morphotypes and rarely matched 100% with any single group. Among the 80 female AZ ♀/GA ♂ F1 hybrids examined, 67 (83.8% (74.1, 90.3%, $\alpha = 0.05$)) had a brown central scutal area that reached the posterior margin of the scutum as in the description for the Arizona *A. maculatum* s. l. (morphotype III) (Figure 2.7, j), and 13 (16.3%

(15.9,16.5)%, $\alpha = 0.05$)) demonstrated scutal coloration defined by a brown central area that did not reach the scutal posterior edge, as in the description for *A. maculatum* s. s. (morphotype II) (Figure 2.7, g).

Hybrid males possessed comma-shaped spiracular plates as described for *A. maculatum* s. l. (morphotype III) (Figure 2.7, i and l). The tibial armatures for male and female hybrids were consistently of unequal thickness, though there was a range of thicknesses of the smaller spur, similar to those identified for both the Arizona *A. maculatum* s. l. (morphotype III) and *A. triste* s. s. (morphotype I) (Figure 2.7, h and k).

Discussion

In the 175 years since Koch first described *Amblyomma maculatum*, there has been no consensus on how to easily define ixodid tick species (Dantas-Torres 2018, Goddard et al. 2019). While morphology is arguably the most practical tool in separating specimens, the characters that distinguish valid tick species are often subjective and difficult to decipher for even trained entomologists. This can become nearly impossible when trying to identify immature stages; nymphs and larvae of *A. maculatum* s. s. and *A. triste* s. s. are rarely collected and not readily distinguished using contemporary morphological keys (Mertins et al. 2010, Mukherjee et al. 2014, Cohen et al. 2015). Molecular analysis can be an effective tool to separate valid species and identify potentially cryptic species, however a biological approach is perhaps the most robust method of elucidating species relationships in Ixodidae (Goddard et al. 2019).

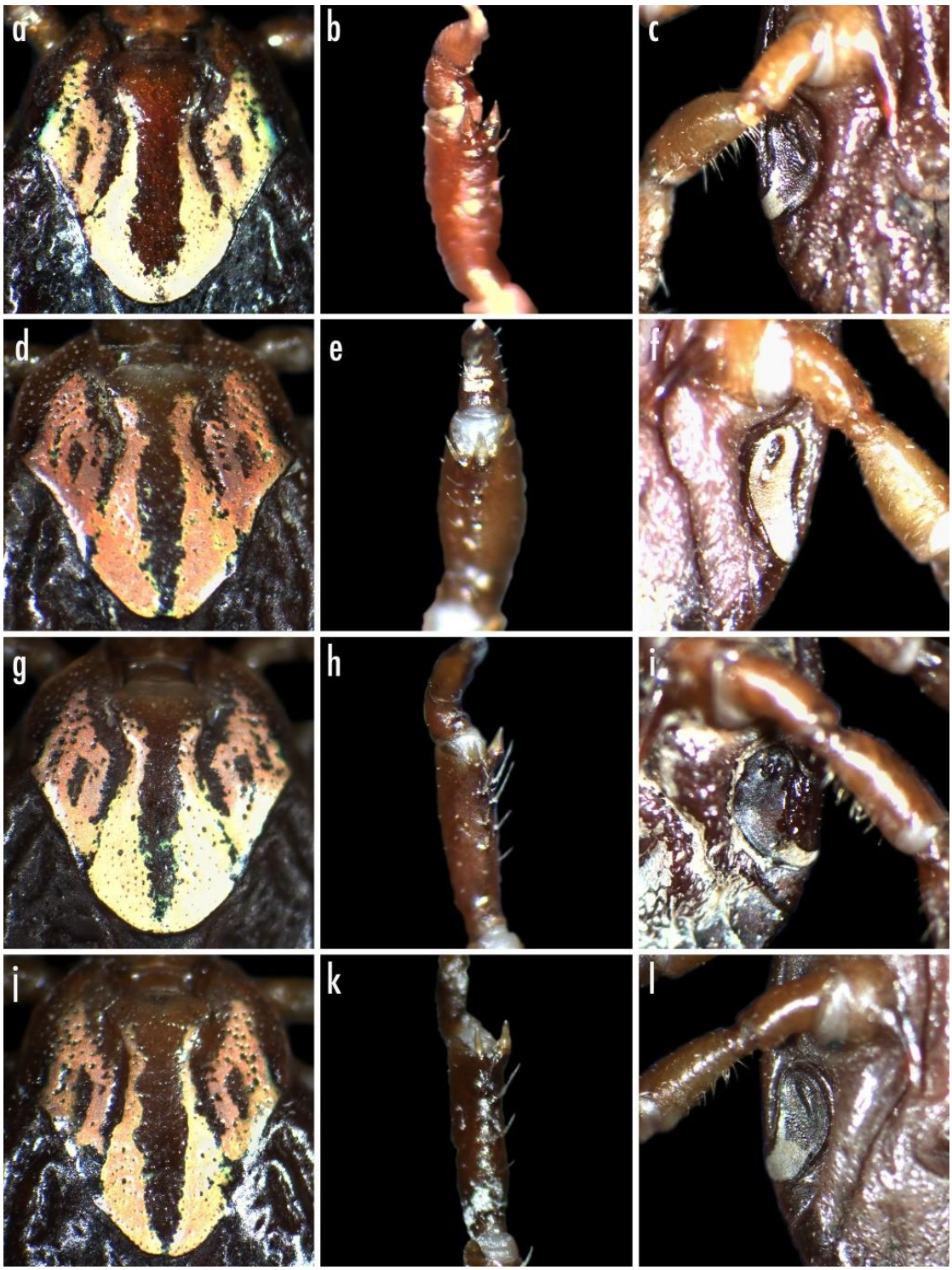

Figure 2.7 Morphology of F1 adults

Images of the morphology of the scutum, armatures on tibiae II – IV, and male spiracular plates for the GA ♀/GA ♂ (a – c), AZ ♀/AZ ♂ (d – f), GA ♀/AZ ♂ (g – i), and AZ ♀/GA ♂ (j – l) F1 adults

The most basic principle of the biological species concept is reproductive isolation, or an accumulation of reproductive isolating mechanisms sufficient to prevent successful gene flow between two populations of closely related organisms (Mayr 1970). This principle has been used to verify species identities within ixodid ticks through crossbreeding experiments many times before. Interspecific studies between *Dermacentor variabilis* (Say), *D. occidentalis* Marx, and *D. andersoni* Stiles (Oliver et al. 1972), *Rhipicephalus (Boophilus) microplus* (Canestrini) and *Rh. (Boophilus) decoloratus* (Koch) (Spickett and Malan 1978), and *D. marginatus* Sulzer and *D. reticulatus* (Fabricius) (Zahler and Gothe 1997) produced no viable F1 hybrids. Similar interspecific crossbreeding analyses between the *Ixodes ricinus* (Linnaeus) – *I. persulcatus* Shulze complex (Balashov et al. 1998) and *Rh. (Boophilus) annulatus* (Say) and *Rh. (Boophilus) microplus* (Canestrini) (Graham et al. 1972) produced infertile F1 hybrids. In both cases, these studies support the validity of these taxa as separate species.

Intraspecific analyses have also been conducted to test conspecificity, such as between the tropical and temperate lineages of *Rh. sanguineus* (Latreille) (Levin et al. 2012) and geographically distinct populations of *Rh. (Boophilus) microplus* (Labruna et al. 2009), *Amblyomma cajennense* (Fabricius) (Labruna et al. 2011), temperate *Rh. sanguineus* s. l. (Dantas-Torres et al. 2018), and *Amblyomma parvum* Aragão (Nava et al. 2016). With the exception of *A. parvum* and temperate *Rh. sanguineus* s. l., all of these intraspecific analyses resulted in reproductive incompatibility, to suggest that the taxa represent multiple biological species.

In the present study, *A. maculatum* s. s. from Georgia and *A. maculatum* s. l. from Arizona effectively hybridized when placed together on animals in a laboratory setting. However, these F1 hybrids did not produce fertile progeny and were unable to establish an F2

generation. Though the F1 hybrids readily recognized each other as mates, they exhibited diminished fitness, most notably seen in the GA ♀/AZ ♂ cohort's apparent inability to feed to repletion across multiple attempts. It is thus likely that one or both of the F1 hybrid sexes is infertile.

Hybrid sterility serves as a postzygotic reproductive isolating mechanism to prevent the exchange of genes between populations, thus preserving the genetic integrity of species. Almost invariably, hybrid sterility follows Haldane's rule of scarcity, preferential impairment, or infertility of the heterogametic sex (Haldane 1922). In *Amblyomma* species, as in most ixodid ticks, males are heterogametic (Oliver 1989) and would be expected to be rare or infertile if Haldane's rule holds true. Such was the case in the attempted crosses of *Amblyomma americanum* (Linnaeus) with *A. maculatum* s. s. and *Amblyomma variegatum* (Fabricius) with *Amblyomma hebraeum* Koch performed by Gladney and Dawkins in 1973 and Clarke and Pretorius in 2005, respectively. In the first experiment, only the cross of *A. maculatum* s. s. males and *A. americanum* females produced F1 offspring, all of which were female (Gladney and Dawkins 1973). The second study showed similar results, with the cross of male *A. variegatum* and female *A. hebraeum* producing F1 progeny that were entirely female (Clarke and Pretorius 2005). In the work shown here, 5/6 of the individual F1 hybrid lineages produced more females than males at ratios from 1.05 – 1.96, with the highest female-to-male ratio in the AZ ♀/GA ♂ hybrid cohort. This cohort is statistically significantly different from the F1 homologous cohorts, although the homologous cohorts also exhibit female-to-male ratios over 1 (Table 2.2). Nonetheless, we did not cross F1 hybrid males with F1 homologous fertile females, so it remains uncertain if Haldane's rule of sterility applies. Similarly, without performing the reciprocal cross

of the F1 hybrid females with fertile F1 homologous males, we cannot know if the hybrid females are potentially the sterile sex.

Having said that, the two allopatric populations of *A. maculatum* group ticks presented here lack the genetic divergence necessary to be classified as separate species (Lado et al. 2018). The adults can be separated morphologically, but characters to do so are slight and somewhat subjective. There is inevitably a gradient of morphological variation within populations of individual species, and although the few characters separating *A. maculatum* s. s. and *A. maculatum* s. l. might otherwise be attributed to this gradient, our observation of reproductive isolation past the F1 generation precludes consideration of these disjunct populations as a single biological species (Mayr 1970). However, while the two populations of ticks in this study from southern Arizona and Georgia appear to represent distinct biological species, more work is required to accurately determine the taxonomic status of *A. maculatum* s. l. ticks identified in the American southwest, including crossbreeding experiments with *A. triste* s. s. from South America. Investigations into variable markers such as microsatellites could further provide clarity to the species relationships between these and other populations within the *Amblyomma maculatum* group (Fagerberg et al. 2001). As more cases of *R. parkeri* rickettsiosis are identified (Yaglom et al. 2020) and the recognized range of this pathogen's *Amblyomma* vector extends in the southwestern United States (Hecht et al. 2020, Paddock et al. 2020), it is vital to determine whether ticks in this region represent a previously described or a unique species.

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CHAPTER III

DEVELOPMENT OF 14 POLYMORPHIC MICROSATELLITE LOCI FOR *AMBLYOMMA MACULATUM*

Abstract

Fourteen polymorphic microsatellite loci were developed for analysis of the Gulf Coast tick, *Amblyomma maculatum* Koch, 1844. This tick is a notable human biter and important vector of *Rickettsia parkeri*, an agent of spotted fever rickettsiosis, in the Western Hemisphere. Microsatellites were designed to amplify *A. maculatum* sensu stricto (s. s.) as well as *A. maculatum* sensu lato (s. l.), a recently identified vector of *R. parkeri* in the southwestern United States with a range that extends from northern Mexico to western Texas. Panels of 15 individual tick lysates were used to identify polymorphisms within these microsatellite loci. The number of alleles per locus ranged from 3 to 15, and single locus heterozygosities ranged from 0.4 to 0.87. Application and analysis of these microsatellite markers will increase our understanding of gene flow within and between *A. maculatum* s. s. and *A. maculatum* s. l., thus helping to clarify the species identities for these ticks as well as other closely related members of the *A. maculatum* group.

Introduction

In 1844, Koch described six species of *Amblyomma* (Acari: Ixodidae) ticks that would be grouped together to form the *Amblyomma maculatum* tick group: *Amblyomma maculatum* from North America, and *Amblyomma triste, Amblyomma tigrinum, Amblyomma ovale, Amblyomma*

ovatum, and *Amblyomma rubripes* from South America. Later morphological analyses would identify *A. rubripes* as a synonym for *A. maculatum* and *A. ovatum* as a synonym for *A. tigrinum* (Kohls 1956), while molecular analyses based on 16S suggest that *A. ovale* is too genetically distant to be considered a member of the *A. maculatum* group (Estrada-Peña et al. 2005). Thus, since 2005 the recognized species in the *A. maculatum* group have been *A. maculatum*, *A. triste*, and *A. tigrinum*.

All three species of the *A. maculatum* group are important vectors of *Rickettsia parkeri*, a pathogen distributed widely across the Americas that causes a mild rickettsiosis in humans (Paddock et al. 2008). In South America, *A. triste* and *A. tigrinum* are the primary vectors (Venzal et al. 2004, Pacheco et al. 2006, Nava et al. 2008, Monje et al. 2014, Romer et al. 2020), with human cases reported from Uruguay (Pacheco et al. 2006, Conti-Díaz et al. 2009, Portillo et al. 2013) and Argentina (Romer et al. 2011, Romer et al. 2014, Villalba Apestegui et al. 2018, Armitano et al. 2019, Romer et al. 2020). In North America, all human cases of *R. parkeri* rickettsiosis have been reported from the United States, with cases from 11 states: Arizona, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Texas, and Virginia (Paddock and Goddard 2015, Herrick et al. 2016, Yaglom et al. 2020).

Historically, in the United States, *A. maculatum* sensu stricto (s. s.) was considered the sole vector for *R. parkeri*, with human *R. parkeri* rickettsiosis cases occurring exclusively within the recognized range of this tick across the eastern and Gulf Coast states (Figure 3.1) (Paddock and Goddard 2015). However, the understanding of the epidemiology of this rickettsial pathogen changed in 2014 with the discovery of two *R. parkeri* rickettsiosis cases from southern Arizona (Herrick et al. 2016). The ticks involved were originally identified as *A. triste*; however, additional morphological analyses determined that ticks from southern Arizona share characters

of both *A. triste* and *A. maculatum* s. s., precluding a definitive morphological assignment to either species (Allerdice et al. 2017, Lado et al. 2018). Hence, since 2017 these ticks have been designated as *Amblyomma maculatum* sensu lato (s. l.).

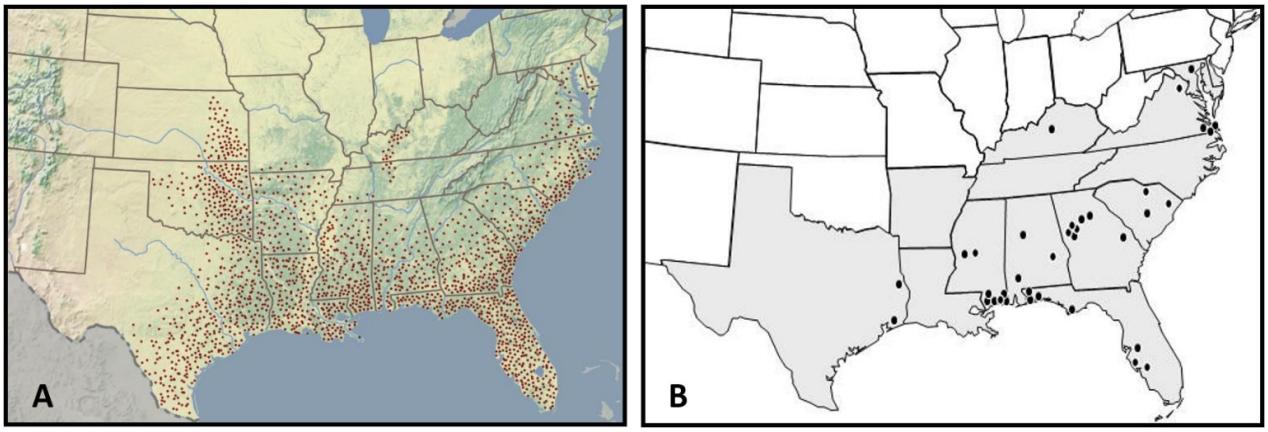

Figure 3.1 *Amblyomma maculatum* s. s. distribution

Image shows **A:** Recognized range of A. maculatum s. s. in 2015 (Paddock and Goddard 2015) and **B:** counties with recognized cases of R. parkeri rickettsiosis between 2004-2014. Image credit Dr. Christopher Paddock, CDC

Rickettsia parkeri-infected *A. maculatum* s. l. have subsequently been identified in additional locations in the southwestern United States, including other sites in Arizona (Allerdice et al. 2017, Hecht et al. 2020), as well as in New Mexico (Hecht et al. 2020) and western Texas (Paddock et al. 2020). Since the initial human cases of *R. parkeri* rickettsiosis in 2014, several additional cases have been reported from Arizona (Yaglom et al. 2020). *Rickettsia parkeri*-infected *A. maculatum* s. l. have also been identified in the northern state of Sonora, Mexico, though no human cases have yet been reported from this country (Delgado-de la Mora et al. 2019).

Efforts to clarify the species identity of the *A. maculatum* s. l. ticks from the southwestern United States have produced mixed results. A comprehensive morphological analysis of the *A. maculatum* group identified four morphotypes within *A. triste* and *A. maculatum,* with *A. maculatum* s. l. from the southwestern United States and northern Mexico representing a unique morphotype distinct from both *A. triste* and *A. maculatum* s. s. (Lado et al. 2018). This same study also included a robust molecular analysis using both mitochondrial and nuclear markers. Results of this molecular analysis indicate that there is insufficient genetic divergence to separate *A. maculatum* s. s. from *A. maculatum* s. l. as individual species (Lado et al. 2018). This study further suggests conspecificity between *A. triste* and *A. maculatum.* In short, this work suggests that *A. triste* and *A. maculatum* could either represent four individual species based on morphology or a single species based on phylogenetic analysis.

To further test the species identity of *A. maculatum* group ticks from the southwestern United States, a cross-mating analysis was performed with *A. maculatum* s. l. from Arizona and *A. maculatum* s. s. from Georgia (Allerdice et al. 2020). This analysis showed that *A. maculatum* group ticks from Arizona and Georgia will mate on experimental animals and can produce F1 hybrid ticks. However, these hybrids apparently cannot successfully produce viable F2 offspring (Allerdice et al. 2020), thus suggesting that *A. maculatum* s. l. from Arizona and *A. maculatum* s. s. from Georgia represent distinct biological species.

The *A. maculatum* s. l. ticks in the southwestern United States have thus far been subject to three species tests: morphologically and molecularly in Lado et al. 2018 and biologically in Allerdice et al. 2020. Results of these direct tests have offered no clear and consistent delineation for these tick populations. A better understanding of the population genetics of *A. maculatum* s. s. and *A. maculatum* s. l. may offer insight into the species identity for these ticks, as well as

provide a better understanding of the migration of these ticks on the local level in the southwestern United States.

Microsatellites are simple nucleotide repeats that consist of motifs repeated in tandem. Several studies have utilized microsatellites to characterize populations of ixodid ticks of medical importance, including *Ixodes scapularis* Say (Ludwig 2015, Leo et al. 2017, Talbot et al. 2020), *Amblyomma aureolatum* (Pallas) (Ogrzewalska et al. 2016), and *Ixodes ricinus* (Linnaeus) (Delaye et al. 1998, de Meeûs et al. 2002, de Meeus et al. 2004). In the present study, we describe the development of polymorphic microsatellite loci for *A. maculatum* s. s. and *A. maculatum* s. l.

Methods

Identification and selection of microsatellites

DNA of nine *A. maculatum* s. s. ticks from different areas of the eastern United States was extracted using the Qiagen Blood & Tissue kit (Qiagen, Valencia, CA, USA). Purified DNA was sent to MrDNA (Shallowater, TX) for whole genome amplification to increase the amount of DNA obtained through the extraction. This was followed by generation of an Illumina pair-ended shotgun library. Illumina paired-end sequences were analyzed using PAL FINDER v0.02.03 (Castoe et al. 2012, Griffiths et al. 2016) to search and identify perfect di, tri, and tetra-nucleotide tandem simple sequence repeats (SSRs) characterized by at least six repeats and amplifiable by primers with GC content greater than 30%, melting temperatures of 58–65 °C with a maximum 2 °C difference between paired primers, and with the last two 3′ nucleotides as G or C.

Primers for microsatellite amplification were synthesized at CDC's Core Facility in Atlanta, GA.

Optimization of polymerase chain reaction (PCR) and gel electrophoresis

All PCR reactions were performed using an Eppendorf Mastercyler® Nexus thermal cycler (Eppendorf AG, Germany), and the initial PCR optimization was performed using Taq Master Mix (Qiagen, Valencia, CA, USA). Individual PCR reactions for this optimization included 10 µL of Taq, 1 µL each of forward and reverse primers at 20 µM, 1 µL of genomic DNA, and 7 µL of PCR-grade nuclease free water to bring the reactions to a final volume of 20 µL.

A touchdown PCR protocol was designed and utilized for the amplification of the microsatellite loci. The protocol was designed to begin the first cycle of amplification at an annealing temperature of 62 °C followed by a step down of 1 °C for each of 14 cycles to end at 48 °C annealing. A 20 cycle repeat at 48 °C annealing with a 72 °C extension step ended the annealing portion of the protocol. The touchdown PCR protocol with all denaturation, annealing, and extension steps can be found in Table 3.1.

Table 3.2 Touchdown PCR protocol for microsatellite amplification

Cycle	Repeats	Step	Dwell time (min)	Hold	Setpoint (°C)
1	1	1	5:00		95
2	14	1	0:30		95
		2	0:30		62.0 – 48.0
		3	0:30		72
3	20	1	0:30		95
		2	0:30		48
		3	0:30		72
4	1	1	10:00		72
5	1	1		X	4

The initial microsatellite screening using this touchdown protocol was performed using a template of pooled DNA that included aliquots from a total of 27 individual *A. maculatum* s. s.,

A. maculatum s. l., and *A. triste* s. s. (Table 3.2). Resultant PCR products from initial pooled DNA screenings were electrophoresed on 4% agarose gels stained with 0.1 mg/ml ethidium bromide. These gels were run at 60 V for 90 minutes to allow ample time for separation of DNA. After electrophoresis, the gels were visualized on a Bio-Rad Gel Doc™ EZ Imager (Bio-Rad, Hercules, California, USA). Microsatellites were identified as potentially informative if they exhibited 2 – 3 clear bands after visualization of the pooled DNA amplicons.

Table 3.3 List of ticks used in the initial pooled DNA PCR screening for microsatellites

Tick species	**Collection location**	**Year Collected**	**Number of ticks**
***Amblyomma maculatum* s. s.**	Delaware, USA	2017	2
	Florida, USA	1996	3
	Georgia, USA	2015	3
		2017	3
	Illinois, USA	2019	2
	Kansas, USA	2013	3
	Mississippi, USA	2011	3
	Oklahoma, USA	2012	2
***Amblyomma maculatum* s. l.**	Arizona, USA	2016	3
***Amblyomma triste* s. s.**	Buenos Aires, Argentina	2007	3
		TOTAL:	**27**

Microsatellites that appeared to be potentially informative after the initial pooled DNA screening were further analyzed using individual tick lysates. A panel of eight A. maculatum group ticks was selected based on geographic diversity to be used in this follow up PCR screening. These included six A. maculatum s. s., with one each from Florida (1999), Georgia (2015), Kansas (2013), Mississippi (2006), North Carolina (2010), and Oklahoma (2013), and two A. maculatum s. l. with one each from Arizona (2016) and Sonora, Mexico (2016). DNA from these individual ticks was amplified using the master mix formulation and cycling

conditions as described above. The resultant amplified PCR products from these individual tick screenings were electrophoresed and visualized as described above to check for polymorphisms between individual ticks. Microsatellites that produced 1 – 2 bands during gel electrophoresis for each individual tick were selected for capillary electrophoresis, with priority given to those microsatellite loci that exhibited differences in band sizes between the individual ticks.

Amplification and capillary electrophoresis of selected microsatellites

Microsatellites identified in the previous steps were further analyzed using capillary electrophoresis. The forward primers were re-synthesized at CDC's core facility with a FAM fluorescent tag on the 5' end, and master mixes for the reactions using these fluorescently tagged primers utilized the Type-it® Microsatellite PCR kit (Qiagen, Valencia, CA, USA). Each reaction consisted of 12.5 μL Type-it®, 2.5 μL each of the tagged forward and untagged reverse primer at 2.5 μM, 1 μL genomic DNA, and 6.5 μL PCR-grade nuclease free water, for a final reaction volume of 25 μL. Cycling conditions were identical to the touchdown protocol described above and illustrated in Table 3.1.

One μL of the resultant tagged PCR product was subsequently added to a mix of 8.75 μL HI-DI™ formamide (Applied Biosystems, Waltham, Massachusetts, USA) and 0.25 μL GeneScan™ 500 ROX™ dye Size Standard (Applied Biosystems, Waltham, Massachusetts, USA) for capillary electrophoresis. Samples were denatured at 95 °C for 5 minutes and then placed on ice until loaded for electrophoresis. All 10 μL of the mixture were loaded onto an ABI 3500 genetic capillary analyzer (Applied Biosystems, Waltham, Massachusetts, USA) for capillary electrophoresis.

Data analysis

Resultant trace files from capillary electrophoresis were uploaded into the GeneMarker® v4.0 (SoftGenetics, State College, Pennsylvania, USA) genotyping software for analysis. Trace files were analyzed to identify null alleles and stuttering as well as to check for polymorphisms. Microsatellites were considered informative if they appeared to have low or no null alleles as well as a mix of allelic expression between the samples tested.

Final microsatellite selection

Polymorphic microsatellite markers identified through the processes described above were subjected to a final analysis using an additional seven tick lysates, bringing the total number of ticks tested for each locus to 15 (Table 3.3, Table 3.5). Single locus heterozygosity was calculated as the proportion of individual samples exhibiting heterozygosity at the locus relative to the total number of samples tested.

Results

The initial search identified over 600,000 microsatellite sequences in the A. maculatum sequencing reads. These were separated by monomer sequence of the motif. To successfully amplify the identified SSR loci and score their alleles, it is important to select loci that will successfully generate PCR products. It is thus essential that primers designed in the flanking regions of the loci amplify a single locus. Therefore, we selected forward and reverse primers observed only once in the entire read dataset, or where one of them was observed once and the other no more than twice. A first selection identified 146 amplifiable loci to be tested on individual specimens.

Table 3.4 List of samples used in the individual screenings for the microsatellites

Sample	Species	Collection Location	Collection Year
AZ-1	*A. maculatum* s. l.	Arizona, USA	2016
AZ-2	*A. maculatum* s. l.	Arizona, USA	2016
FL-1	*A. maculatum* s. s.	Florida, USA	2007
FL-2	*A. maculatum* s. s.	Florida, USA	1996
FL-3	*A. maculatum* s. s.	Florida, USA	2007
GA-1	*A. maculatum* s. s.	Georgia, USA	2015
GA-2	*A. maculatum* s. s.	Georgia, USA	2015
KS-1	*A. maculatum* s. s.	Kansas, USA	2013
KS-2	*A. maculatum* s. s.	Kansas, USA	2013
KS-3	*A. maculatum* s. s.	Kansas, USA	2013
MS-1	*A. maculatum* s. s.	Mississippi, USA	2003
MS-2	*A. maculatum* s. s.	Mississippi, USA	2011
MS-3	*A. maculatum* s. s.	Mississippi, USA	2020
MS-4	*A. maculatum* s. s.	Mississippi, USA	2020
MS-5	*A. maculatum* s. s.	Mississippi, USA	2020
MS-6	*A. maculatum* s. s.	Mississippi, USA	2020
MX	*A. maculatum* s. l.	Sonora, Mexico	2017
NC	*A. maculatum* s. s.	North Carolina, USA	2010
NM	*A. maculatum* s. l.	New Mexico, USA	2019
OK-1	*A. maculatum* s. s.	Oklahoma, USA	2013
OK-2	*A. maculatum* s. s.	Oklahoma, USA	2013
OK-3	*A. maculatum* s. s.	Oklahoma, USA	2013

A total of 138 of these 146 microsatellite loci were analyzed using the pooled DNA template and touchdown PCR protocol (Table 3.4). While microsatellites with at least six repeats were initially prioritized for PCR optimization, additional loci with fewer than six repeats were included in the pooled DNA PCR testing after encountering some amplification problems with some of the microsatellite loci.

Of the 138 loci tested, 23 microsatellites produced gel images that appeared to contain 2 – 3 bands and were subjected to further testing with the initial panel of eight individual ticks as described above. Gel electrophoresis and visualization of these loci yielded 17 microsatellites

with both homozygous and heterozygous expression for the individual ticks based on the presence of one or two bands (Figure 3.2). These 17 loci were screened with an additional seven tick lysates so that the total samples screened for each locus included four *A. maculatum* s. l. samples (two from Arizona and one each from New Mexico and Mexico) and 11 *A. maculatum* s. s. samples (two each from Florida, Georgia, Kansas, Mississippi, and Oklahoma and one from North Carolina) (Table 3.5).

Table 3.5 Microsatellite loci tested

Locus Tag	Motifs	# of repeats	Forward Primer	Tm (°C)	Reverse Primer	Tm (°C)
1	AGT	7	CGGTAAATTCGGTAGACTATAGGG	59.1	AAATACAGGGCCATGAAATGC	59.3
2	AGT	8	CGTCGAATTCGGTAGACTATAGGG	61.6	ACCATGGCTCCTTCATAGCG	60.1
3	AT	8	TTCAATAAAGCATGTTGCCG	57.4	TAGAGTGGTCACGAGAGGCG	58.4
4	AT	9	TGATAATTCTCATGATCTAGCGTGC	60.8	TTGAACAGGGAGTAAGCGGG	60.2
5	AT	10	TTCTAGTGGTATGCAATCAACTCC	58.3	AACAAGAAGGGAGCAGAAAACC	59.6
6	AT	11	AAACTTGACGAAGCCAAGGG	59.4	GCGACCATGTGGTGACTCC	58.7
7	AT	13	CAGCAATATACTGGGAAGCCC	59.1	CCAGCTGGTTCACCTGTACG	58.7
8	ATC	8	AGTGATCATGACACCCGTCG	58.4	TCAACGACCATACCAATGCC	58.9
9	ATC	10	TGCAAAACGTGGAACTAAAAGG	60.0	TGCAGTGGGAATAACCAGGC	60.7
10	ATC	11	CAGCACTCATCGCTACAGGC	59.1	TGGATGAATACGGGAGAGGC	59.7
11	ATC	12	CAACATCCTCAGAGCCCTCC	59.2	TTCCGCCATATGTTTCCTCC	59.9
12	ATT	6	GTAATAAGATGGCGGCAGGC	60.0	TGTCCAGCCTTGTCTTGTCC	58.0
13	ATT	7	GTGTGCTAGCCGGTTCTTCC	59.7	GGTGCGCCATTTGATGC	58.8
14	ATT	8	CTTCGATTGGTGCAAACTGG	58.9	TACTTCGAAGCAACCCCTGC	60.3
15	ATT	9	TCGCAACCAATACAACTCTGC	58.8	GAAGGCGAAACGAAGTAGCG	60.3
16	CG	8	CCTTCGCTATTCACACTCCG	58.3	CCCCAATAGGCTCATGTAACG	59.8
17	CG	8	TTCACCCGAGATTAGCGAGC	60.4	TGGGGTAGTGAGTGTGAGCG	59.0

Table 3.5 (continued)

18	CGG	5	ACTTGCCTCACGGTTTAGCC	59.4	CTAAAATTTCGGCTGCCTCC	59.4
19	CGG	6	GGGACGCGTAGTAAGCAAGG	60.1	CCGCACGTCAACACACTACC	59.4
20	AAC	27	AATTCGGCTTCCGTTTAGGG	61.0	CGACCCATCTAGGAGCAAGG	59.6
21	AC	8	TGGAAAGTTTGTTTGCGAAGC	60.7	CAGTGAGCCTGTCTTCGACC	57.7
22	AC	8	CGTACAATTCACAGGGGCG	59.5	AAGACAAGGGTCAGCTTCGC	59.4
23	AC	9	ACCACCACCCACACAAATCC	59.8	CGCACTCTGATTCTGGATCG	58.8
24	AC	10	CGTACAATTCACAGCACCCC	58.6	ATGAGGAGACAAGAACCCGC	58.9
25	AC	11	TCAATTCTTTCACACAAACAGACG	59.8	TGAAAAGACGCCTACCGAGC	60.5
26	AC	13	GCCATATTCCCACAGACTGC	57.7	AGCGCAAGATGCATTACACC	58.7
27	ACC	6	GGTAAATTCGACGCAATGCC	60.6	AAATCCCATAATCCCCACCC	60.0
28	ACC	7	CTTTGCGCAGAAATTGAAGG	58.8	TTTGGGTGTCGCTGTGTAGC	59.3
29	ACC	8	TTCACCGTTTTCGTGTCAGG	59.1	CTGCTCAAATAGGCCTTGGG	59.9
30	ACC	10	TGATAATTCGACGCAATGCC	59.5	GCAAACATTACCCATTCCCC	59.2
31	ACC	14	TGTTCAGTGCGACTTCACCC	58.9	TAGAGCCTGGGCCAATAGGG	61.7
32	CGG	7	CGGTAAATTCCAAGCAATAACCC	62.2	GCGCTATCAAACCTCTTATCCG	61.1
33	CGG	8	ACAATTCTGCCACACGTTCG	59.1	AAGAGGGTGGAGCCGAGC	59.7
34	CGG	9	CCGGGTGAGGTGTATGTCC	57.8	GCTGCTCCCACACTTGACG	59.4
35	TC	8	TTGGAGACCGGGTAGTTTCG	60.0	GGGGCTAGAATGGCCTCC	59.1
36	TC	9	GGTTAATTTTAAGACACCGCCG	61.0	GAAGGAAAATGCCACTGCG	58.9
37	TC	10	CGTCACAAAATTTGCTTCGG	59.3	TTTGGCACGATTTTCAGACG	59.5
38	TC	12	ACAATTTGGGGTGGCATAGG	59.7	CGAAACCGAAGCCTACTTGC	60.2
39	TC	13	TGCTTTTGTAACGTACGGCG	60.1	CCTTCATGCCTTTCTCTCGC	59.9
40	TC	14	CTTTGCAACATCATGGTCCG	59.5	TGGAGGCCTTTCACATAGGG	59.9
41	TCC	6	CGCAAGTAGATGGAATGGGG	60.3	GTTAACCCCTCAGCCGTTCC	61.0
42	TCC	7	TCGTTGCATGATTGGAAAGC	59.4	ATGCGGTAGAGTCCTGGTGC	59.8
43	TCC	8	ATTTGACATCCCTGCCTTCC	58.8	ACGTGAAACCTTGAGCTCCG	60.1
44	TCC	9	TTTCGCAGTATGTCCAAGCG	59.7	GTCTAGCTCCCTCCCTTTCG	58.2

Table 3.5 (continued)

45	TCC	13	CGTGCAATTCCGCTCG	56.7	GTGTGCGACTTGGCTACTGC	59.0
46	TCG	6	CGGTAAATTCGCTCTCAACG	58.6	CCTGAGCGAGTGATACGTGC	58.9
47	TCG	7	TTGGTATCTTGGAGATGGCG	58.4	AGGTGGGAGAAGAAAATGCG	59.3
48	TCG	8	ATTCTTCCAGAGTGGGTGGG	58.6	CAAAGAAAATTCCTTGCGCC	60.2
49	TCG	13	CGAATTCTGTCGTGTCCTCG	58.7	AGTTAATGCGTCCCTTTCGC	59.6
50	TGC	6	TATGCGTATACGGCACCAGG	59.5	CACGTGCGCATAAACATCG	59.1
51	TGC	7	ACCATCTTTATCATCCCGGC	58.9	ATCGTGTTGGAGGGAAAACG	59.8
52	TGC	9	CCAGTTGCTGTGCCTCACC	59.2	ATACGACGACGCATCACACG	60.0
53	TGC	10	TGCTTGGCACACTCTGACG	58.2	CATTTGAGCGTGACTCGTCC	58.7
54	TTC	6	CAGTCAATTCTTGGTGGAAGC	57.3	TCTATCAAGCACCCCAATGC	58.4
55	TTC	7	CCTACGGTTATTCCGCTTCG	60.1	ACCGTTGCGAATCTTGTCG	59.2
56	TTC	8	CTTTCCCCTAGTGGCAGTGG	59.6	GGCGCAAGGAAGGAATAGG	59.8
57	TTC	9	TTTTCTATTGCCATTCCCGC	60.3	GGGAAGAGCCACATTTGACC	59.2
58	TTC	11	TACGCAGTCGATGAATTCGC	59.6	TCTTTATTCCGTTGTGCGCC	61.2
59	TGC	3	AAAGTCCGTCCACATCCTGC	59.6	CCGCGAGTAATGGGGTAGC	60.5
60	TCC	3	TACGACATTCAAACGCGACC	59.4	AACGTTCGTCCTCGTGGC	58.8
61	TTC	3	GCCCACTGTTGATATGTGCC	58.4	CGAGCACTCCTTCCCATACC	59.4
62	TCG	3	GACAGAGTTCGACGCTAGGC	57.4	GTGTGTGTCCGAGAAGCAGC	58.6
63	ATT	3	CGTACAATTCGCGTACAGGG	59.2	GAGTCGCAGGCATTTACTGG	58.2
64	AGT	3	GAGGCATCGTTTCGATTTCC	59.6	AGGTATGCTAGCAGCCACGC	60.5
65	AGGT	7	CGGTAAATTCTCTCCCGACC	58.7	GGACGCTCTTTCAGTTTGTGC	59.9
66	AGTG	5	GTAAATTCAGGTCTGGGCCG	59.6	TTCGCACATTACATCAGCCG	60.3
67	AGTG	6	TTGGAATTCTGCTCTTCAGCC	59.6	CCTGGAAGCATGACACATCG	59.3
68	ATAC	4	TTGTAATTCCCAGAGAGGGG	56.6	TTGTTTTGTGAGTAATGTGCGG	59.0
69	ATAC	6	AACACATCTGGGCATTGACG	58.8	ATATACACACCTCGCCGTGC	58.4
70	ATAC	10	TTGCCCAGAGCTAATACCCG	60.4	CGACATTTCATTGTGCTGCC	59.6
71	ATCG	4	ATCGGTCTCAGATTCTCCGC	58.9	GTGCTTTTCTAGCGTTCGGG	60.2

Table 3.5 (continued)

72	ATCT	4	TCTACATTGTATGTTAGACACCAGGC	59.9	AAACCAGACTGCAGGAACGG	59.9
73	ATCT	5	CGGAAGATACCGTGCTCAGG	60.5	GGCCAACCTCTCTTCTTTTCC	59.7
74	ATGC	4	AGCCCTAAAAGCCAAAAGCC	60.6	CACATCAGCGTATGTGTGTGC	57.8
75	ATGG	4	TTCACCGAATGAAATGGTGG	58.9	TATCTTCGCTCAATGCCAGC	58.9
76	ATGG	5	GCTCCGTGCTGATTTAACCC	59.7	GACCACTGTCTCGTAGCCCC	59.2
77	ATGG	6	CGTCGAATTCGTTAGTCTGGC	60.0	CGATGTCAGAGCACGTTAAGC	58.5
78	ATGG	7	TGTGCAACACAAACACAAGAGG	59.4	TTGGAACTTCCTCAAACGCC	60.1
79	CCCG	4	CTGATGTCAATCCTGCCACG	59.3	GAGTGCCATACACCATTGCC	58.4
80	CCCG	5	CCCCATTTACCTGTTGGAGC	59.4	ATCATTGCATGCGTCTGTCG	60.0
81	TCCG	4	CCGTCGGTCTGTCAGTCG	57.1	GTGATGTAACGTTGCCCTGG	58.6
82	TCCG	5	CACCGATTCTTCCATATCCTCC	60.2	GGCTTCAACCGCGTCC	57.2
83	TCCG	9	GATACTCCGTCTTCGGTGCC	59.4	GCGCTAGCACAAAACATTCC	58.6
84	TCTG	4	ATCGGTAGATCGGTTCACGG	59.7	CCATTGAAGACGAAAGCTGC	58.4
85	TCTG	4	CGTCTCGGTCGTCTTGTGC	59.4	TGTCTGCCTTGATTTATTTCCG	59.7
86	TCTG	5	ATGGACCGGCATACATTTGG	60.4	CACCTTCGTTTTGCTGTTGG	59.0
87	TGCC	4	CGCTCTCAACACAAAATGCC	59.1	CTGCACTGGTGAAGGCTAGG	58.2
88	TGCC	5	GAATTCAGAGCTCCCTTCGC	59.0	ATAAGATTCAGTGCGCCACG	58.7
89	TGCG	4	TCTGCAGCACTACACAACGC	57.9	TTTGTCGCTCCATTCGGG	59.8
90	TGCG	4	AGTCATATTTGGGTTGGCCC	58.8	ACCTTTGCCTGGTAGTTCGC	59.4
91	TGCG	6	TGCAGAATACGTGGTTTCGG	59.4	CGCCTACCAACCTTCTCAGG	59.7
92	TGGG	4	CCAGTACGGCGACAAGAAGG	60.6	CAACCACCACCGCCCC	60.2
93	TGGG	5	AACAAACGAAACGGGAGAGG	59.2	AAATTCGATGGGATTGCAGG	59.9
94	AAAC	4	AAAATCAGTTTCGGATCGGG	59.2	GGTGCTTATTTCTGAAAGCCG	59.5
95	AAAC	5	ACTAACCTGGAGCGACTGCG	60.3	AAACACAAAAGCAACTGGCG	58.9
96	AAAC	6	CAAGAACTCCGAAGGGATGG	59.4	TGGTCACCTATGGACTCCCG	60.3
97	AAAC	7	CAAAGCGTATTAGTGAACCGC	58.1	TGCTCAAGGCTACGAAGACG	59.0
98	AAAG	4	GGCTGAAGACGAGGAGTTCG	59.7	TCAGAGCTAAGCCGATGACG	58.9

Table 3.5 (continued)

99	AAAG	5	CGAATTCCATTTATTACAGTTTGTTCG	63.1	TTCAGGTGAAATAGTGAAGCGG	59.8
100	AAAG	6	GATAGCGCAGTGTCACACCC	58.5	CGGATCGAAGCACTGGG	57.3
101	AAAG	8	TACAGGCTCTCCACGGTTGC	60.9	GTGCTCGAGGGATTAGTGGG	59.4
102	AAAT	4	TAGGCTTTCGGTTGAGAGCG	60.7	AAACCGTCCTCTAAAGGGGC	60.0
103	AAAT	5	CCTGCAATAAACGAGTGCCC	60.6	GGATCAGAGTTGGACACCGC	59.9
104	AAAT	7	GAACGCTATTTGGTGATGCG	59.2	CATAGGGGCACAATTTTAGCG	60.3
105	AACG	8	GTACTGGTCGGAGCAAGCG	58.9	CGGCCTGAAAGAGGTCG	56.7
106	AACG	11	TATGGCGACTTGCATTTTCG	59.8	CCAAACTCTGGACTGACCTGC	59.5
107	AAGC	4	CGGTAAATTCTTTATTTTCTTCCACC	61.8	TGCTGGATTGTCTGTACCCG	59.0
108	AAGC	5	CGTGCTCCCCAATAATACCC	59.5	TCTCCCTACGACCATGAGAGG	59.0
109	AAGC	7	AGATTTCGCCAACCCATACG	60.0	GCTTCCCTTCAGCTTCTTCG	59.3
110	AAGT	5	GAATTCATTGCTGGAACCCC	59.2	CTGTCAACGGATCACAAGCC	58.5
111	AATC	4	GGTTTCGCATTCAACAGTCG	58.9	TGCGTCCACATATTGTTCCC	58.9
112	AATC	7	GATGTATCATCCGTCGTGCC	58.3	TGTTTCGGACTGTTAAGCCC	57.8
113	AATC	9	TGATAATTCGTGCAATGGCG	60.4	TGCCAGATCAGAACGAATGC	59.2
114	AATG	4	GCGATTTCTTAGCGGTTTCG	60.4	GCTATCTGGCGCAGTCACC	58.7
115	AATG	5	AGAGCACTTACCGGCTCACC	59.2	GCTGAGCAGTTTCCTCGGG	60.4
116	AATG	9	GGAAGCGCGTTGTCTTCG	59.8	GGTAGTCGCTGCTCCAGCC	60.5
117	AATT	4	ATCAGTGACTAAGCCGTGCG	58.7	TATTGTTCACCCAAGCTCCC	57.4
118	AAC	27	AATTCGGCTTCCGTTTAGGG	61.0	CGACCCATCTAGGAGCAAGG	59.6
119	ACCG	4	TGTAGAGCCAGTTATGCCGC	58.9	GGCATGATTGTAAATGATTAACGC	61.0
120	ACGT	6	ACCGTCAGCTTTGTCAGTGC	58.3	AGCGCAGGACCTATTCATCC	59.2
121	ACTG	4	TGTGTTTGACGCAGAGGAGC	59.3	ACGTCGCAGAACACTTGACG	59.3
122	ACTG	5	CACCTGTTTCGCTTCAATGG	58.9	AAGCACTGGCGAACACACC	58.9
123	AGCC	4	CGCCAGCCATTAAAACACC	59.0	GGTCGTCCTGACTGCATGG	58.9
124	AGCC	6	TTGGAATTCGTATGGTATTTGC	57.5	TTGACGCTGCAACACTCG	56.5
125	AGCG	4	TGAGTGAGGAGTGAGCGAGC	58.3	TGGCCGGTATAGGTTCAAGG	60.1

Table 3.5 (continued)

126	AGCT	4	AATTCAAGCGCGGAGGC	59.3	AGTGGGACCTGACCTTAGCG	59.1
127	AGCT	4	GTAAATTCAAGCGCGGAGG	58.5	GTTGGAAGGCGATTATGACG	58.1
128	AGGG	4	GATAGGGTTCCCTTTTGCGG	61.1	TTCAGGTCAAGGGATGTTGC	58.1
129	AGGG	6	GGGGTAGCTCCAGAGTAGCG	59.1	AAGGGAGTTTAAAAGGCGGG	60.3
130	AGGT	4	ACAACTACGCTTAGCCGACG	58.3	CCCCTCCGAGAATAACCC	56.5
131	TTCC	4	GTGTGGACATCTGAATCGCC	58.1	GTGCACTGAACACCGTACCC	58.3
132	TTCC	5	GATGTCCGGAGGTCTGTAGC	56.7	AGACAAGGCGCAGTATCTACC	56.5
133	TTCC	5	AGCGAGCTCTCCATACGTGG	60.2	CTGATGATGGTGGGATGTGG	58.6
134	TTCC	6	AGAGCATCCATGGGTGAAGG	59.5	CGACGGTACAGGAATAGGGC	59.6
135	TTGG	4	CAATTCGAAGGAAATAAGAAAATACAC	63.0	CGGTAGTATCTCGGGCAAGG	59.8
136	TTGG	5	TTCCCTGACATCCTGAGTGC	57.8	GGAGAGTGTTTGCTCCGTCC	59.3
137	TTGG	6	GACCTTTACAAATGCACAAATGC	59.4	GAGGAAAATGAGCCCACTCG	59.5
138	TTGG	7	TGCCAACAGACAACCTTTGC	59.3	CGACCACTGCTTTAACGTGC	59.2

These screenings resulted in the identification of 14 polymorphic loci for the tick lysates tested, including loci 5, 12, 19, 20, 36, 53, 56, 74, 91, 95, 103, 112, 114, and 119 as seen in Table 3.4. Individual alleles for each sample can be seen in Table 3.5. The years of collection for each sample listed in Table 3.5 can be found in Table 3.3; because of a lack of sample volume and potential DNA degradation for the older samples, it was not possible to use the same 15 ticks for all 14 loci.

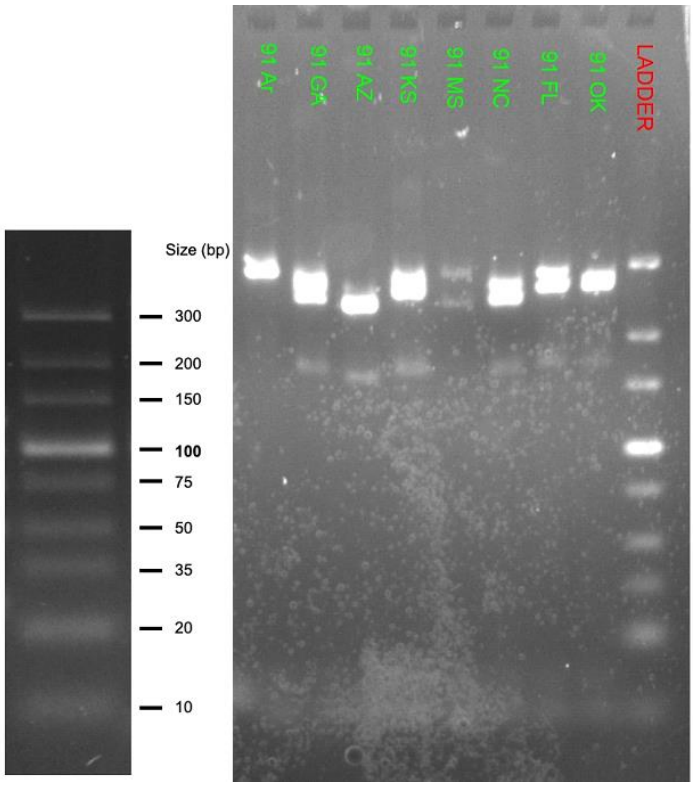

Figure 3.2 Sample microsatellite agarose gel

Sample gel showing allelic expression of microsatellite 91. Heterozygosity is illustrated by the presence of two bands between 200 – 300 bp. Homozygotes exhibit a single band within this size range.

Individual locus heterozygosity ranged from 0.4 in loci 20 and 74 to 0.87 in loci 12 and 91 (Table 3.5), and the average heterozygosity for the 14 loci was 0.63 [95% CI (0.52, 0.73)]. The total alleles per locus ranged from three in locus 12 to 15 in locus 56 (Table 3.5), and the average alleles per locus for all 14 loci was 7.9 [95% CI (6, 9.7)].

Table 3.6 Results of individual microsatellite screenings

Locus Tag:	5	
Repeat:	2 bp	
Alleles:	7	
Heterozygosity:	0.8	
AZ-1	380	386
AZ-3	380	380
FL-1	378	382
FL-3	376	380
GA-1	376	380
GA-2	374	376
KS-2	384	386
KS-3	374	378
MS-3	380	382
MS-4	380	382
MX	380	382
NC	378	382
NM	378	378
OK-2	376	376
OK-3	376	380

Locus Tag:	12	
Repeat:	3 bp	
Alleles:	3	
Heterozygosity:	0.87	
AZ-1	430	439
AZ-3	430	439
FL-1	439	469
FL-3	439	469
GA-1	439	469
GA-2	439	469
KS-2	469	469
KS-3	439	469
MS-1	469	469
MS-3	439	469
MX	430	439
NC	439	469
NM	430	439
OK-2	439	469
OK-3	439	469

Locus Tag:	19	
Repeat:	3 bp	
Alleles:	6	
Heterozygosity:	0.73	
AZ-1	236	245
AZ-2	236	245
FL-1	248	248
FL-2	224	245
GA-1	236	245
GA-2	236	245
KS-2	236	245
KS-3	248	248
MS-2	245	245
MS-3	236	245
MX	236	245
NC	248	248
NM	236	245
OK-2	254	263
OK-3	248	254

Locus Tag:	20	
Repeat:	3 bp	
Alleles:	10	
Heterozygosity:	0.4	
AZ-1	187	190
AZ-2	190	190
FL-1	211	211
FL-2	211	229
GA-1	211	211
GA-2	235	235
KS-1	232	262
KS-2	235	235
MS-1	262	262
MS-2	205	214
MX	190	190
NC	205	214
NM	187	187

Locus Tag:	36	
Repeat:	2 bp	
Alleles:	4	
Heterozygosity:	0.53	
AZ-1	317	317
AZ-2	317	317
FL-1	303	327
FL-2	331	331
GA-1	303	327
GA-2	303	327
KS-1	303	327
KS-2	303	327
MS-3	327	327
MS-2	327	327
MX	317	317
NC	303	327
NM	317	317

Locus Tag:	53	
Repeat:	3 bp	
Alleles:	9	
Heterozygosity:	0.6	
AZ-1	266	266
AZ-2	266	266
FL-1	260	272
FL-2	266	278
GA-1	284	284
GA-2	263	287
KS-1	278	290
KS-2	278	278
MS-1	260	266
MS-2	272	275
MX	266	266
NC	260	287
NM	266	266

Table 3.6 (continued)

OK-1	238	238
OK-2	214	229

Locus Tag:	56
Repeat:	3 bp
Alleles:	15
Heterozygosity:	0.47

AZ-1	288	288
AZ-2	327	336
FL-1	243	279
FL-2	327	336
GA-1	291	291
GA-2	267	267
KS-1	228	276
KS-2	228	285
MS-1	222	309
MS-2	243	243
MX	288	288
NC	297	297
NM	327	327
OK-1	285	315
OK-2	282	282

Locus Tag:	95
Repeat:	4 bp
Alleles:	4
Heterozygosity:	0.43

AZ-1	500	500
AZ-2	500	500
FL-1	492	492
FL-2	502	506
GA-1	492	500
GA-2	492	492
KS-1	492	492
KS-2	492	500
MS-5	492	492
MS-2	**	**
MX	500	500
NC	492	500

OK-1	303	327
OK-2	303	327

Locus Tag:	74
Repeat:	4 bp
Alleles:	7
Heterozygosity:	0.4

AZ-1	357	373
AZ-2	357	357
FL-1	277	277
FL-2	277	277
GA-1	277	325
GA-2	277	325
KS-1	277	325
KS-2	329	329
MS-1	277	277
MS-2	277	277
MX	361	361
NC	277	277
NM	353	357
OK-1	277	277
OK-2	277	325

Locus Tag:	103
Repeat:	4 bp
Alleles:	10
Heterozygosity:	0.67

AZ-1	386	386
AZ-2	390	434
FL-2	396	396
FL-3	392	404
GA-1	386	386
GA-2	396	404
KS-1	388	396
KS-3	386	412
MS-2	384	396
MS-3	392	396
MX	392	392
NC	384	392

OK-1	263	287
OK-2	275	275

Locus Tag:	91
Repeat:	4 bp
Alleles:	11
Heterozygosity:	0.87

AZ-2	246	250
AZ-3	246	258
FL-2	246	258
FL-3	274	290
GA-1	278	278
GA-2	266	266
KS-2	246	262
KS-3	266	286
MS-2	266	274
MS-3	266	274
MX	258	280
NC	250	254
NM	246	258
OK-2	266	274
OK-3	266	274

Locus Tag:	112
Repeat:	4 bp
Alleles:	7
Heterozygosity:	0.73

AZ-1	267	267
AZ-2	267	275
FL-1	267	267
FL-2	267	275
GA-1	279	279
GA-2	271	271
KS-1	275	283
KS-2	267	275
MS-1	279	287
MS-2	263	279
MX	267	275
NC	271	279

Table 3.6 (continued)

NM	500	500
OK-1	492	492
OK-2	492	500

Locus Tag:	114
Repeat:	4 bp
Alleles:	7
Heterozygosity:	0.47

AZ-1	304	304
AZ-2	292	304
FL-1	312	312
FL-2	312	312
GA-1	292	292
GA-2	284	296
KS-1	312	312
KS-2	308	308
MS-2	300	304
MS-3	292	304
MX	304	304
NC	292	308
NM	296	304
OK-1	312	312
OK-2	292	308

NM	386	434
OK-2	392	400
OK-3	396	396

Locus Tag:	119
Repeat:	4 bp
Alleles:	10
Heterozygosity:	0.8

AZ-3	246	258
AZ-2	278	286
FL-2	258	258
FL-3	258	258
GA-1	250	258
GA-2	258	258
KS-2	246	258
KS-3	246	258
MS-2	258	302
MS-3	262	266
MX	278	290
NC	246	258
NM	246	258
OK-2	250	270
OK-3	246	258

NM	267	275
OK-1	267	275
OK-2	267	283

The two columns of numbers represent the alleles for the individual samples. Heterozygotes are defined as those with two different alleles for an individual sample, and homozygotes are defined as those with a single allele for an individual sample. The years of collection for the samples are available in Table 3.3.

Discussion

In this study, we identified and optimized 14 polymorphic microsatellite loci that can be used in future population genetics studies for *A. maculatum* s. s. and *A. maculatum* s. l. Microsatellite markers developed here should allow for investigation of a diverse range of questions about *A. maculatum* on many spatial scales, from the local population level to cross continental and intercontinental analyses.

The *A. maculatum* s. l. tick populations in the southwestern United States and northern Mexico are closely tied to the Madrean Occidental archipelago sky islands (Warshall 1995, Allerdice et al. 2017, Delgado-de la Mora et al. 2019, Hecht et al. 2020, Paddock et al. 2020). The Madrean Sky Islands are areas of subtropical woodland habitats in the mountain ranges of southeastern Arizona, southwestern New Mexico, and northern Mexico. These sky islands are surrounded by the Sonoran and Chihuahuan deserts. Because of the arid environment outside the riparian sky islands where *A. maculatum* s. l. are found, ticks from these areas likely depend on migratory birds, cattle, or other vertebrates who can traverse long distances to disperse tick specimens to additional suitable sky island habitats within the surrounding desert. Notably, the vertebrate hosts for *A. maculatum* s. l. have not been elucidated. Longitudinal investigations into patterns of gene flow between populations of *A. maculatum* s. l. in the sky islands of the southwestern United States and northern Mexico may offer insight into the movement of their vertebrate hosts, thus providing clues as to the identity of those hosts.

Multi-locus sequence typing analysis of *Rickettsia parkeri* indicates that there may be a tight relationship between one distinct genotype and *A. maculatum* s. l. in the southwestern United States (Allerdice et al. 2021). That study identified four North American *R. parkeri* genotypes, three of which exhibit broad geographic dispersal (Figure 3.3), even for those with relatively few samples. However, the genotype represented in Clade V is limited entirely to the southwestern United States within the range of *A. maculatum* s. l. While there is another genotype present in the southwestern United States and northern Mexico (Figure 3.3), it is clear that *R. parkeri* is evolving on the local level within the range of *A. maculatum* s. l.

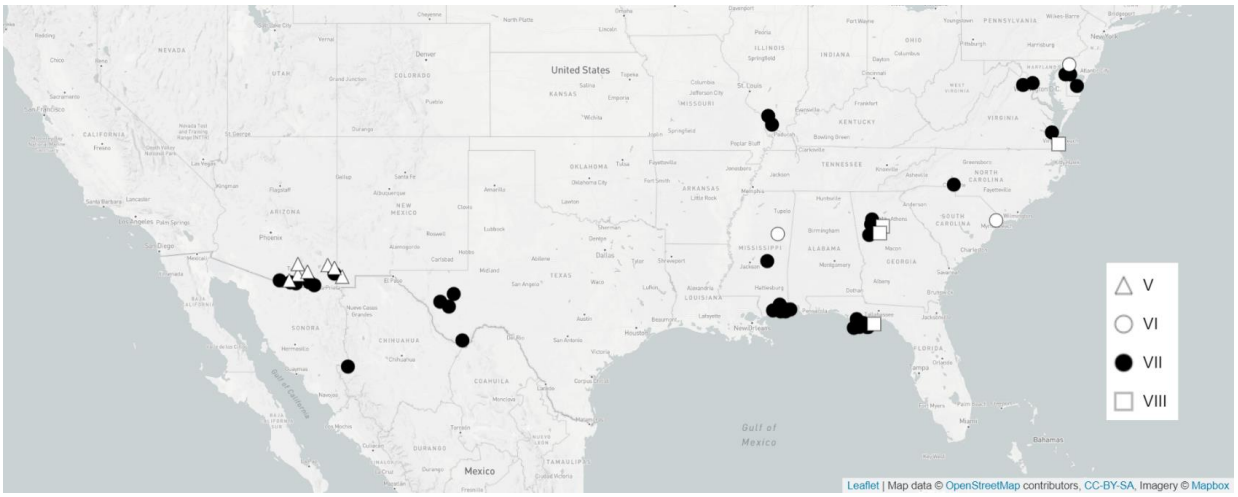

Figure 3.3 Map showing the distribution in North America of *R. parkeri*

Clade 5 is indicated by white triangles, Clade 6 by white circles, Clade 7 by black circles, and Clade 8 by white squares. From Allerdice et al. (2021).

The coevolution of rickettsial organisms with their tick vectors has been proposed before (Azad and Beard 1998, Krawczak et al. 2018), with recent studies providing evidence for speciation of *Rickettsia* within their tick vectors in the Neotropics (Estrada-Peña et al. 2021). Investigations into the population genetics of *A. maculatum* s. l. using the microsatellite markers developed here could offer clarity on local population differences where this unique *R. parkeri* genotype is found. These analyses could perhaps provide insight on whether or not coevolution between *R. parkeri* and *A. maculatum* s. l. is occurring, similar to what has been suggested for the Neotropics.

As mentioned previously, a cross-mating analysis between *A. maculatum* s. l. from Arizona and *A. maculatum* s. s. from Georgia resulted in reproductive incompatibility (Allerdice et al. 2020), while molecular analyses indicate that these two tick populations are conspecific (Lado et al. 2018). The microsatellite markers developed here can be used in comprehensive population genetics studies of the *A. maculatum* group across its range in both North and South

America. To truly understand species relationships between *A. maculatum* s. s. and *A. maculatum* s. l., a broader study that includes these tick species' nearest relatives can provide a baseline of genetic relatedness against which individual populations can be compared.

The systematics of the *Amblyomma maculatum* group have been redefined several times since Koch's original description of this group. While analyses and species tests for individuals within the group have been performed numerous times, a population analysis of all members at once has yet to be completed. It is our hope that microsatellite markers developed here can be used in such an analysis to not only provide clarity on species identities of ticks within the *A. maculatum* group, but also to elucidate evolutionary relationships between *A. maculatum* s. l. and *Rickettsia* and provide evidence to clarify the ecology of these ticks in unusual habitats such as the southwestern United States and northern Mexico.

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CHAPTER IV

PHYLOGENETIC DIFFERENTIATION OF *RICKETTSIA PARKERI* (RICKETTSIALES: RICKETTSIACEAE) REVEALS BROAD DISPERSAL AND DISTINCT CLUSTERING WITHIN NORTH AMERICAN STRAINS

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Abstract

The tick-borne pathogen *Rickettsia parkeri* causes a mild rickettsiosis, with cases reported from several countries throughout its known distribution in the Americas. Molecular analyses have identified a clear distinction between strains of *R. parkeri* sensu stricto (s. s.) and *R. parkeri* sensu lato (s. l.) as well as separation between North American and South American *R. parkeri* s. s. strains. To expand on this previous work, we developed a multi-locus sequence typing analysis with two aims: first, to investigate the genetic diversity within strains of North American *R. parkeri* s. s. and second, to further understand the genetic relationships between *R. parkeri* s. s. and *R. parkeri* s. l. Sixty-four *R. parkeri* isolates and 12 *R. parkeri*-positive tick lysates were analyzed using a novel typing scheme consisting of four coding regions and two intergenic regions. A concatenated Bayesian phylogeny was constructed that identified eight clades: three represent the *R. parkeri* s. l. strains and five represent the *R. parkeri* s. s. strains. The clades appear to be generally phylogeographically organized and associated with specific

tick vectors. However, while one of the four *R. parkeri* s. s. North American clades appears to be limited to the southwestern United States, the other North American clades exhibit broad dispersal, most notably seen in the largest group which includes representative samples extending from northern Mexico to Delaware. This work highlights the increasingly recognized geographic range of *R. parkeri* in the Americas and suggests a potential public health risk for these areas.

Introduction

The genus *Rickettsia* consists of obligately intracellular bacteria, many of which are human pathogens transmitted via arthropod vectors including mites, fleas, and ticks (Yu and Walker 2006). Until the early 2000s, *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), was the only tick-associated rickettsiosis recognized in the United States. However, in 2002 a rickettsiosis caused by *Rickettsia parkeri* was confirmed in a patient from Virginia, thus establishing *R. parkeri* as the second tick-associated rickettsiosis in the United States (Paddock et al. 2004). Human cases of *R. parkeri* rickettsiosis resemble those of other spotted fever group rickettsioses but are characteristically less severe than RMSF; there have been no known fatalities associated with *R. parkeri* rickettsiosis. The incubation period ranges from 2 – 10 days post exposure, and the disease is most commonly characterized by an inoculation eschar at the site of tick attachment. Other symptoms in humans are less specific, but often include a combination of fever, headache, malaise, and myalgia (Paddock et al. 2008, Biggs et al. 2016). Since the initial case, more than 50 cases of *R. parkeri* rickettsiosis have been identified, all limited to the Americas (Venzal et al. 2004, Nava et al. 2008, Conti-Díaz et al. 2009, Romer et al. 2011, Portillo et al. 2013, Paddock and Goddard 2015, Herrick et al. 2016, Straily et al. 2016, Faccini-Martínez et al. 2018, Yaglom et al. 2020).

The primary vectors of *R. parkeri* in North America are the ixodid ticks *A. maculatum* sensu stricto (s. s.) in the eastern and Gulf Coast regions of the United States and *A. maculatum* sensu lato (s. l.) in southern Arizona (Teel et al. 2010, Paddock and Goddard 2015, Herrick et al. 2016, Straily et al. 2016, Yaglom et al. 2020). Recent field studies further identified *R. parkeri* sensu stricto (s. s.) in *A. maculatum* s. l. ticks in West Texas (Paddock et al. 2020) and New Mexico (Hecht et al. 2020), as well as in the northern state of Sonora (Delgado-de la Mora et al. 2019) and the southeastern state of Tabasco, Mexico (Torres-Chable et al. 2020). In South America, *Amblyomma triste* Koch, 1844 and *Amblyomma tigrinum* Koch, 1844, both members of the *A. maculatum* tick group (Estrada-Peña et al. 2005), are the primary vectors of *R. parkeri* s. s. (Venzal et al. 2004, Pacheco et al. 2006, Nava et al. 2008, Monje et al. 2014, Romer et al. 2020). Cases have been reported from Uruguay (Pacheco et al. 2006, Conti- Díaz et al. 2009, Portillo et al. 2013, Faccini-Martínez et al. 2018) and Argentina (Romer et al. 2011, Romer et al. 2014, Villalba Apestegui et al. 2018, Armitano et al. 2019, Romer et al. 2020), where these ticks are prolific human biters.

Although ticks in the *A. maculatum* group are recognized as primary vectors for *R. parkeri* s. s., other closely related strains of this pathogen have been described in other tick species. In South America, the Atlantic rainforest strains of *R. parkeri* sensu lato (s. l.) have caused four confirmed cases of human rickettsiosis in Brazil, with *Amblyomma ovale* Koch, 1844 implicated as the responsible vector (Spolidorio et al. 2010, Silva et al. 2011, Krawczak et al. 2016, da Paixão Sevá et al. 2019). A recent report further confirmed a case of human rickettsiosis caused by *R. parkeri* s. l. strain Atlantic rainforest from Colombia (Arboleda et al. 2020) in a region where an isolate of this pathogen was previously obtained from a questing *A. ovale* tick (Londono et al. 2014). An additional strain of *R. parkeri* s. l. Atlantic rainforest was

isolated from *Amblyomma aureolatum* (Pallas, 1772) in southern Brazil in the state of Santa Catarina, where *R. parkeri* s. l. strain Atlantic rainforest had previously been detected in *A. ovale*, *A. aureolatum*, and *Rhipicephalus sanguineus* (Latreille, 1806) (Medeiros et al. 2011, Barbieri et al. 2014).

Several strains of *R. parkeri* s. l. not associated with human illness have also been identified in the Americas. Rickettsial isolates closely related to *R. parkeri* s. l. Atlantic rainforest have been recovered from *Amblyomma nodosum* (Neumann, 1899) in Brazil (Ogrzewalska et al. 2009) and *Amblyomma parvitarsum* Neumann, 1901 (Ogrzewalska et al. 2016) in Argentina and Chile. While *A. parvitarsum*, *A. nodosum*, *A. ovale*, and *A. aureolatum* are not known to exist in the United States, a recent study reported detection of a *Rickettsia* species most closely related to the Brazilian *R. parkeri* s. l. Atlantic rainforest strains recovered from *A. ovale* ticks in Veracruz, Mexico (Sánchez-Montes et al. 2019), confirming this pathogen's presence in North America. Additionally, the recently characterized *Rickettsia parkeri* s. l. strain Black Gap has been reported from the United States and northwestern Mexico in the tick *Dermacentor parumapertus* Neumann, 1901 (Paddock et al. 2017, Sánchez-Montes et al. 2018). Strain Black Gap is most closely related to the South American Atlantic rainforest strains. While animal experimentation suggests that this strain could be a human pathogen, no human cases have been reported to date. However, coupled with the broad range of *D. parumapertus* in western North America, the close relationship between strain Black Gap and the *R. parkeri* s. l. Atlantic rainforest strains suggests a potential public health risk associated with this organism.

The public health implications of *R. parkeri* have expanded significantly since its initial isolation from *A. maculatum* in 1937 (Parker et al. 1939). As our knowledge of this emerging

pathogen has progressed, so has our understanding of its relationship with its ixodid tick vectors. While the original isolates and human infections of *R. parkeri* were all associated with *A. maculatum* s. s., in recent years this pathogen and several closely related strains have been detected in a wide range of confirmed and potential tick vectors. Recent genetic analyses based on both coding and non-coding regions identified South American and North American groupings of *R. parkeri* that could be subdivided based on their tick vectors (Nieri-Bastos et al. 2018). However, that study was based on relatively few *R. parkeri* s. s. strains, and notably did not include any *A. maculatum* s. l. rickettsial strains from the southwestern United States or Mexico. While this analysis clearly shows genetic separation between North and South American *R. parkeri* s. s. isolates, the 13 North American isolates included in the study represent only four U.S. states and exhibit very little genetic variation based on the eight molecular targets analyzed. The majority of human *R. parkeri* rickettsiosis cases have been reported from the United States; thus, to better characterize the genetic divergence within North American strains of *R. parkeri* s. s., we describe herein a comprehensive multi-locus sequence typing analysis for an extensive panel of isolates of *R. parkeri* s. s. and *R. parkeri* s. l., with inclusion of tick lysates for regions in which isolates are not available.

Methods

All 50 of the North American *R. parkeri* s. s. and *R. parkeri* s. l. isolates used in this study are part of the Centers for Disease Control and Prevention's Rickettsial Isolate Reference Collection. The 14 South American isolates were kindly provided by Drs. David Walker (Necocli_10_11) and Marcelo Labruna (Agua Clara, At10, At24, Pantanal At46, At5URG, Parvitarsum Ar, Parvitarsum Ch, NOD Pantanal, Atlantic rainforest strains Paty, Ao10, Ao2240, Adrianópolis, and Aa46). North American tick samples for the study were provided by Dr.

Victoria Phillips (Illinois), Drs. Lauren Maestas and Michael Buoni (Delaware), and Drs. Jesus Delgado, David Delgado and J. David Licona-Enriquez (Mexico). DNA extracts of *R. parkeri*-infected *A. triste* ticks were provided by Dr. Santiago Nava (Argentina).

Rickettsia parkeri s. s. and s. l. isolates (Table 4.1) were propagated in Vero E6 cells in Minimum Essential Medium (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA). Cultures were maintained in a 5% atmospheric CO_2 incubator at 34 °C until 90% infection was observed by visualization with acridine orange staining (Figure 4.1) (BD, Franklin Lakes, NJ, USA). DNA was extracted from the propagated cultures using a KingFisher ML automated purification system (Thermo Scientific, Waltham, Massachusetts, USA). DNAs were eluted in 150 µl of KingFisher elution buffer and stored at 4 ℃ prior to genetic analysis. Species verification was performed using a PCR assay targeting a portion of the rickettsial outer membrane protein *omp*A (Roux et al. 1996). PCR products were gel purified and bi-directionally sequenced on an Applied Biosystems 3500 genetic analyzer using a BigDye Terminator V3.1 kit (Applied Biosystems, Carlsbad, CA, USA). Resultant sequences were assembled in Geneious Prime 2019.1 (www.geneious.com) and compared to GenBank data using BLASTn analysis.

Genome alignments were performed using Geneious Prime. Whole genomes for *Rickettsia parkeri* strains Portsmouth (NC_017044), Grand Bay (NZ_LAOK01000001), Tate's Hell (NZ_LAOO01000001), AT#24 (NZ_LAOL01000001), and Atlantic Rainforest (CP040325) were aligned with a draft genome for *R. parkeri* strain Black Gap (data not shown). Thirty-two homologous intergenic regions and primer pairs previously used in genotyping analyses of *Rickettsia conorii* (Fournier et al. 2004) and *R. rickettsii* (Karpathy et al. 2007) were located in the *R. parkeri* Portsmouth genome and compared to the other five aligned genomes to identify

Table 4.1 Rickettsial samples used for analysis in this study

Country of Origin	Sample	Sample Type	Specific Location	Year of Isolation	Source Material	Reference
Brazil	Agua Clara	Isolate	Água Clara, Mato Grosso do Sul	2008	*Amblyomma triste*	(Nieri-Bastos et al. 2013)
	At10	Isolate	Corumbá, Mato Grosso do Sul	2011	*Amblyomma triste*	Unpublished
	At24	Isolate	Paulicéia, São Paulo	2007	*Amblyomma triste*	(Ogrzewalska et al. 2009)
	Pantanal At46	Isolate	Poconé, Mato Grosso do Sul	2012	*Amblyomma triste*	(Melo et al. 2015)
	NOD Pantanal	Isolate	Nhecolândia, Mato Grosso do Sul	2011	*Amblyomma nodosum*	Unpublished
	Atl. Rain. Paty	Isolate	Chapada Diamantina, Bahia	2014	*Amblyomma ovale*	(Nieri-Bastos et al. 2016)
	Atl. Rain. A010	Isolate	Peruíbe, São Paulo	2010	*Amblyomma ovale*	(Szabó et al. 2013)
	Atl. Rain. Ao240	Isolate	Peruíbe, São Paulo	2010	*Amblyomma ovale*	(Szabó et al. 2013)
	Atl. Rain. Adrianópolis	Isolate	Adrianópolis, Paraná	2014	*Amblyomma ovale*	(Nieri-Bastos et al. 2016)
	Atl. Rain. Aa46	Isolate	Blumenau, Santa Catarina	2011	*Amblyomma aureolatum*	(Barbieri et al. 2014)
Uruguay	At5URG	Isolate	Toledo, Chico, Canelones	2004	*Amblyomma triste*	(Pacheco et al. 2006)
Colombia	Atl. Rain. Necocli_10_11	Isolate	Necoclí	2010	*Amblyomma ovale*	(Londono et al. 2014)
Chile	Parvitarsum Ch	Isolate	Arica and Parinacota	2012	*Amblyomma parvitarsum*	(Ogrzewalska et al. 2016)
Mexico	Am/MX 8M	Tick lysate	Yecora, Sonora	2016	*Amblyomma maculatum* s.l.	(Delgado-e la Mora et al. 2019)
Argentina	AT-75	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	(Nava et al. 2008)
	AT-137	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	(Nava et al. 2008)
	AT-190	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	(Nava et al. 2008)
	AT-193	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	(Nava et al. 2008)
	Parvitarsum Ar	Isolate	Salta	2013	*Amblyomma parvitarsum*	(Ogrzewalska et al. 2016)
United States	Carr Canyon	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	Chiricahua	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	Cochise	Isolate	Cochise County, Arizona	2016	*Amblyomma maculatum* s.l.	(Allerdice et al. 2017)
	Cottonwood Spring	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	Guindani Canyon	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	Portal	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	White Wing Spring	Isolate	Cochise County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)

Table 4.1 (continued)

Arivaca Lake	Isolate	Pima County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
Atascosa Spring	Isolate	Santa Cruz County, Arizona	2016	*Amblyomma maculatum* s.l.	(Allerdice et al. 2017)
Cave Creek Canyon	Isolate	Santa Cruz County, Arizona	2016	*Amblyomma maculatum* s.l.	(Allerdice et al. 2017)
Pajarita	Isolate	Santa Cruz County, Arizona	2017	*Amblyomma maculatum* s.l.	(Allerdice et al. 2017)
Thumb Butte	Isolate	Yavapai County, Arizona	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
6W	Tick lysate	Sussex County, DE	2019	*Amblyomma maculatum* s.s.	(Maestas et al. 2020)
9A	Tick lysate	Kent County, DE	2019	*Amblyomma maculatum* s.s.	(Maestas et al. 2020)
10B	Tick lysate	Kent County, DE	2019	*Amblyomma maculatum* s.s.	(Maestas et al. 2020)
18A	Tick lysate	New Castle County, DE	2019	*Amblyomma maculatum* s.s.	(Maestas et al. 2020)
Apalachicola	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Cash Bayou	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Deep Creek	Isolate	Franklin County, Florida	2012	*Amblyomma maculatum* s.s.	Unpublished
High Bluff	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Longleaf	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Sandbank Creek	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
SR-65	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Tate's Hell	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
TH07-94	Isolate	Franklin County, Florida	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Kennesaw Mountain	Isolate	Cobb County, Georgia	2017	*Amblyomma maculatum* s.s.	(Allerdice et al. 2019)
Coweta	Isolate	Coweta County, Georgia	2014	Human	(Straily et al. 2016)
Arabia Mountain	Isolate	DeKalb County, Georgia	2017	*Amblyomma maculatum* s.s.	(Allerdice et al. 2019)
Sweetwater	Isolate	Douglas County, Georgia	2017	*Amblyomma maculatum* s.s.	(Allerdice et al. 2019)
Moe	Isolate	Rockdale County, Georgia	2016	*Amblyomma maculatum* s.s.	(Allerdice et al. 2019)
110958_D	Tick lysate	Pulaski County, Illinois	2013	*Amblyomma maculatum* s.s.	(Phillips et al. 2020)
110954_A	Tick lysate	Jackson County, Illinois	2013	*Amblyomma maculatum* s.s.	(Phillips et al. 2020)
Bayou Heron	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Escatawpa	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Franklin Creek	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
Grand Bay	Isolate	Jackson County, Mississippi	2010	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)

Table 4.1 (continued)

	I-10	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
	Moss Point	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
	MS07-22	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
	MS07-44	Isolate	Jackson County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
	Oktibbeha	Isolate	Oktibbeha County, Mississippi	2009	*Amblyomma maculatum* s.s.	(Paddock et al. 2010)
	NIAID Maculatum 20^{T}	Isolate	Mississippi	1948	*Amblyomma maculatum* s.s.	(Lackman et al. 1965)
	Animas Creek	Isolate	Hidalgo County, New Mexico	2018	*Amblyomma maculatum* s.l.	(Hecht et al. 2020)
	DP 18-72	Tick lysate	Hidalgo County, New Mexico	2018	*Dermacentor parumapertus*	Unpublished
	NC-15	Isolate	Mecklenburg County, North Carolina	2010	*Amblyomma maculatum* s.s.	(Varela-Stokes et al. 2011)
	NC-3	Isolate	Mecklenburg County, North Carolina	2010	*Amblyomma maculatum* s.s.	(Varela-Stokes et al. 2011)
	NC-8	Isolate	Mecklenburg County, North Carolina	2010	*Amblyomma maculatum* s.s.	(Varela-Stokes et al. 2011)
	Horry-SC2006	Isolate	Horry County, South Carolina	2006	Human	(Paddock et al. 2008)
	Black Gap	Isolate	Brewster County, Texas	2015	*Dermacentor parumapertus*	(Paddock et al. 2017)
	Windmill	Isolate	Jeff David County, Texas	2019	*Amblyomma maculatum* s.l.	(Paddock et al. 2020)
	Madera Canyon	Isolate	Jeff Davis County, Texas	2019	*Amblyomma maculatum* s.l.	(Paddock et al. 2020)
	Chisos Basin	Isolate	Brewster County, Texas	2019	*Amblyomma maculatum* s.l.	(Paddock et al. 2020)
	Ponderosa	Isolate	Jeff Davis County, Texas	2019	*Amblyomma maculatum* s.l.	(Paddock et al. 2020)
	Fairfax	Isolate	Fairfax County, Virginia	2010	*Amblyomma maculatum* s.s.	(Fornadel et al. 2011)
	I-66	Isolate	Fairfax County, Virginia	2010	*Amblyomma maculatum* s.s.	(Fornadel et al. 2011)
	Portsmouth	Isolate	Norfolk County, Virginia	2002	Human	(Paddock et al. 2004)
	Ft. Story	Isolate	Princess Anne County, Virginia	2006	Human	(Whitman et al. 2007)
Zimbabwe	*Rickettsia africae* Z9-Hu^{T}	Isolate	Chiredzi, Masvingo	1992	Human	(Kelly et al. 1992)

polymorphisms within these regions. Primers were modified when necessary for specificity with *R. parkeri* for any intergenic regions with observed polymorphisms between the strains. Additional intergenic regions and coding regions containing polymorphisms within the six aligned genomes were identified and selected for further analysis. Primers for these regions were designed using Geneious Prime.

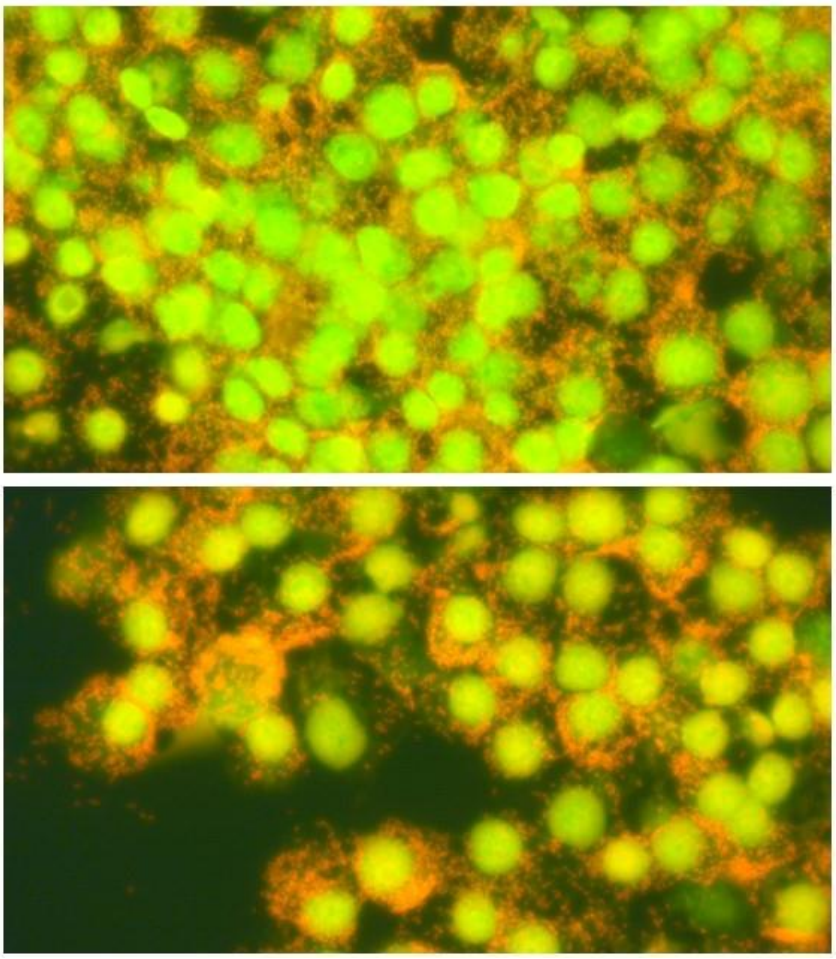

Figure 4.1 *Rickettsia parkeri* in cell culture

Image showing *Rickettsia parkeri* strain Maculatum 20^T in Vero E6 cells stained with Acridine orange. Photo was taken using a fluorescent microscope. Both intra- and extracellular *R. parkeri* can be seen here fluorescing in orange. Photo credit Sandor Karpathy, CDC.

PCR amplifications were conducted in 20 µl reactions, using 10 µl of Taq PCR master mix (QIAGEN, Valencia, CA, USA), 1 µl each of the forward and reverse primers at 20 µM, 2 µl of DNA extract, and 6 µl of nuclease-free water. Reactions were run with an initial 5-minute denaturation at 95 ℃, followed by 40 cycles of a 5-second 95 ℃ denaturation, 45-second annealing step (Table 4.2), and 1-minute extension step at 72 ℃. The final step in the reaction was a single 10-minute extension at 72 ℃. PCR products were gel purified and bi-directionally sequenced as described above. Resultant sequences were assembled using Geneious Prime, and alignment files were constructed in MEGA X (Kumar et al. 2018).

Table 4.2 Primers developed and used for amplification of coding or intergenic regions (IGR) for this analysis.

Locus Tag (*R. parkeri* Portsmouth NC_017044)	Product	Forward Primer	Reverse Primer	Amplicon Size (bp)	Annealing Temp (℃)
MC1_RS05545 - MC1_RS05550	IGR	GTGCAGTTCTGTTGTCATCC	TGCTTGAATGTACCGGAGAA	214	54
MC1_RS03940 - MC1_RS03945	IGR	AGGTGTATACATAAAAGTCTCCA	CTTATCTCTCGCACCTTGGT	139	52
MC1_RS05545	guanosine polyphosphate pyrophosphohydrolase	CTGGATCCCGTGGTCAAGTC	AGATGCCGAGCTTGGTAGAG	296	54
MC1_RS06275	dihydrolipoyl dehydrogenase	TAACACCGCCTGCAAGCTTA	GTTATAGGCGGTGGTCCAGG	273	54
MC1_RS06395	hypothetical protein	TTATTACCCGTGCCGGTTCC	TCTCCACTCCTTCCGGTTCT	321	54
MC1_RS06595	alpha/beta hydrolase family protein	GGGGCTAGTAAACGGTGGTG	AATATTGTAAGCCCGCCGCC	276	54

An initial screening to check for polymorphisms in each locus was conducted using five *R. parkeri* strains that were chosen based on their geographical separation and variation in isolation source. These included strains NIAID Maculatum 20^T (Mississippi, *A. maculatum* s. s.), Black Gap (Texas, *D. parumapertus*), Moe (Georgia, *A. maculatum* s. s.), Cochise (Arizona, *A. maculatum* s. l.), and Atlantic rainforest Aa46 (Brazil, *A. aureolatum*). The five sequences from

this initial PCR screening were added to MEGA X alignments that included GenBank data from the five published *R. parkeri* genomes, creating initial alignments of 10 strains per locus. These alignments were used to identify genetic differences between these 10 strains to determine the potential for discrimination among a larger sample of strains.

For the final analysis, all isolates and tick lysates from Table 4.1 as well as an isolate of *Rickettsia africae* Z9-Hu^{T} were PCR amplified and bi-directionally sequenced as described above according to the annealing temperatures from Table 4.2. *Rickettsia africae* was selected to be used as an outgroup for this work based on its close genetic relationship with *R. parkeri* (Nieri-Bastos et al. 2018). Primer sequences were identified and removed in MEGA X, and insertions and deletions were treated with the simple INDEL coding method (Simmons and Ochoterena 2000). The resultant sequences were assembled and alignments for each locus and the concatenated final analysis were generated with Geneious Prime.

Phylogenetic trees were inferred by the Bayesian method. Bayesian analyses were performed using the MrBayes 3.2.6 program within Geneious Prime (Huelsenbeck and Ronquist 2001). General Time Reversible (GTR) was utilized as the substitution model, and *R. africae* Z9-Hu^{T} was designated as the outgroup. A gamma model of variable rates across sites was used, and 1,100,000 generations were employed with four range categories. Support values for branches are posterior probabilities obtained by MrBayes. Maps of the phylogenies were created using Microreact (www.microreact.org) (Argimón et al. 2016).

Results

A total of 49 loci were assessed with the initial ten-isolate screening panel. Many of these loci clearly separated the *R. parkeri* s. l. strains from the *R. parkeri* s. s. strains. However, because this separation is not novel, loci that demonstrated polymorphisms within *R. parkeri* s. s.

were considered for the final analysis, with priority given to those loci that exhibited differences within the North American *R. parkeri* s. s. strains. Of the 49 loci tested, six were selected for inclusion in the final analysis, including two intergenic regions (*R. parkeri* Portsmouth locus tags MC1_RS05545 - MC1_RS05550 and MC1_RS03940 – MC1_RS03945) and four genes (*R. parkeri* Portsmouth locus tags MC1_RS05545, MC1_RS06275, MC1_RS06395, and MC1_RS06595) (Table 4.2). Partial sequences for these four genes and two intergenic regions were obtained for the 65 rickettsial isolates and 12 tick lysates in Table 4.1.

Of the 77 samples sequenced, 65 were strains of *R. parkeri* s. s., including 54 isolates and 11 tick lysates (Table 4.1). The remaining 11 *R. parkeri* samples were close relatives of *R. parkeri* s. s. and are designated as the *R. parkeri* s. l. samples. These included 10 isolates: Atlantic rainforest strains Paty, Ao10, Ao240, Aa46, Adrianópolis, and Necocli_10_11, strain NOD Pantanal, strain Black Gap, strain Parvitarsum Ar, and strain Parvitarsum Ch. The single remaining tick is DP18-72, a lysate from a *D. parumapertus* tick infected with a rickettsial organism most closely related to strain Black Gap (Table 4.1). The final rickettsial isolate included in the analysis is *R. africae* Z9-Hu^{T}, used as an outgroup and to root the concatenated phylogeny.

Intergenic regions

MC1_RS05545 - MC1_RS05550

Four genotypes exist at this locus and are based on three single nucleotide polymorphism (SNPs) and two different insertion/deletion (INDEL) events (Figure 4.2A). Compared to the reference genome Portsmouth (genotype I), all *R. parkeri* s. s. isolates and tick lysates are identical and comprise one genotype with the inclusion of strain NOD Pantanal. The second genotype for this locus (genotype II) consists of the Atlantic rainforest strains Aa46,

Adrianópolis, Ao240, Necocli_10_11, and Ao10. These strains are identical to each other and have a four base pair insertion (TTAT) beginning at position 31 relative to the reference genome as well as two guanine-to-adenine transitions at sites 44 and 162 relative to the reference. Strain Black Gap and tick lysate DP18-72 comprise the third genotype (genotype III). Both of these samples have the same guanine-to-adenine transitions at positions 44 and 162 and an insertion at the same location in the consensus as the Atlantic rainforest strains; however, these two samples exhibit a smaller two base pair insertion (AT) beginning at position 31 of the consensus amplicon. Genotype IV for this locus consists of the *R. parkeri* s. l. strains Parvitarsum Ch and Parvitarsum Ar. These strains are identical to genotype III except they have a guanine-to-adenine transition at position 96 relative to the reference genome.

MC1_RS03940 - MC1_RS03945

This locus has eight genotypes (Figure 4.2B). The first and second genotypes consist of the *R. parkeri* s. s. samples. North American strains Arabia Mountain, Ft. Story, Moe, and Tate's Hell (genotype II) have a nine base pair deletion at positions 13 – 21 (TCTTTTGTA) relative to the reference genome Portsmouth (genotype I) for this locus, while the remaining 56 North and South American *R. parkeri* s. s. samples (genotype I) are identical to strain Portsmouth. The third genotype (genotype III) contains only strain NOD Pantanal, which has a seven base pair deletion at positions 26 – 32 (TGTATCT), a guanine-to-adenine transition at position 68, and an adenine insertion at position 73 relative to the reference genome. Genotype IV contains Atlantic rainforest strains Ao10, Ao240, and Adrianópolis. This genotype contains the same nine base pair deletion at positions 13 – 21 seen in genotype III, however this genotype also contains an eight base pair insertion (TAAAAAAT) at positions 47 – 54, an adenine-to-cytosine transversion at position 69, and an 11 base pair deletion (AATTATTAAAA) at positions 96 – 106 not seen in

the third genotype. Genotype V contains only Atlantic rainforest strain Necocli_10_11 and is identical to genotype IV except it does not have the adenine-to-cytosine

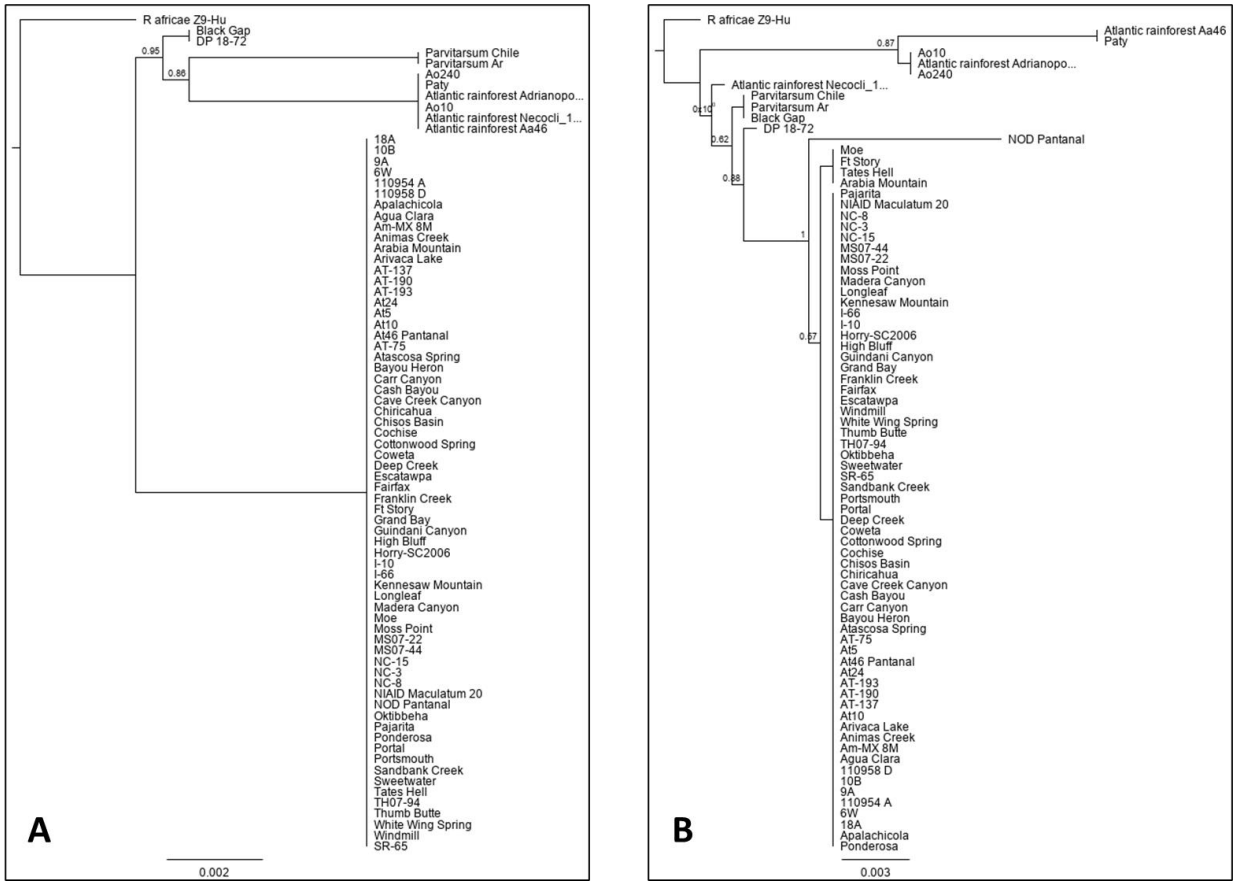

Figure 4.2 Individual phylogenies for intergenic regions

Phylogenies show **A)** the four genotypes for IGR MC1_RS05545 - MC1_RS05550 and **B)** the eight genotypes for IGR MC1_RS03940 - MC1_RS03945.

transversion at position 69. The sixth genotype (genotype VI) consists of the Atlantic rainforest strains Aa46 and Paty. Genotype VI is identical to genotype IV except it has a thymine-to-cytosine transition at position 14. Genotype VII consists of strain Black Gap, strain Parvitarsum Ch, and Parvitarsum Ar, and genotype VIII consists of tick lysate DP18-72. Genotype VII exhibits the nine base pair deletion at positions 13 – 21 seen in genotypes III - VI, however tick

lysate DP18-72 (genotype VIII) does not exhibit this deletion. Both genotype VII and tick lysate DP18-72 (genotype VIII) have an 11 base pair insertion (TAAAAAATTAT) at positions 47 – 57 and the same 11 base pair deletion at positions 96 – 106 seen in genotypes IV – VI.

Coding Regions

MC1_RS05545

This locus has six genotypes (Figure 4.3A). Genotype I consists of 42 of the 50 *R. parkeri* s. s. strains and tick lysates from North America, all identical to strain Portsmouth. Genotype II consists of strain NIAID Maculatum 20^T, which exhibits a thymine insertion at position 198 relative to the reference genome Portsmouth (genotype I). Genotype III contains strain Grand Bay, which exhibits a guanine-to-adenine transition at position 235 relative to genotype I. The fourth genotype (genotype IV) consists only of strain High Bluff, with a guanine-to-adenine transition at position 276 relative to genotype I. Genotype V contains the Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Paty, Ao240, Ao10 as well as Parvitarsum Ar, Parvitarsum Ch, the North American strain Black Gap and tick lysate DP18-72. Also included in genotype V are *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as tick lysates AT-75, AT-137, AT-190, and AT-193. Finally, this group also contains a subset of the North American strains from southern Arizona and New Mexico: Animas Creek, Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring, as well as tick lysate 10B from Delaware. The samples in genotype V all contain an adenine-to-cytosine transversion at position 122 relative to genotype I. The sixth genotype for this locus (genotype VI) contains only strain NOD Pantanal, which contains the adenine-to-cytosine transversion at position 122 seen in genotype V as well as a thymine-to-cytosine transition at position 205 relative to genotype I.

MC1_RS06275

This locus has three genotypes (Figure 4.3B). The first of these genotypes (genotype I) consists of the 50 North American *R. parkeri* s. s. strains and tick lysates, all identical to strain Portsmouth. Genotype II contains Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Paty, Ao240, and Ao10. When compared to the reference genome (genotype I), these strains have two SNPs: a guanine-to-thiamine transversion at position 43 and a thiamine-to-guanine transversion at position 215. The third genotype (genotype III) consists of strains Black Gap, NOD Pantanal, Agua Clara, At24, At5, At10, At46 Pantanal, Parvitarsum Ar, and Parvitarsum Ch as well as tick lysates DP18-72, AT-75, AT-137, AT-190, and AT-193, all of which exhibit the same transversion at position 43 as genotype II; however, they do not possess the SNP at position 215.

MC1_RS06395

This locus consists of four genotypes (Figure 4.4C). The first genotype (genotype I) for this locus consists of 45 North American strains and tick lysates, all identical to strain Portsmouth. Genotype II for this locus consists of the South American *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as the South American tick lysates AT-75, AT-137, AT-190, and AT-193. Also included in genotype II are a subset of the North American strains from southern Arizona and New Mexico: Animas Creek, Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring. This second genotype exhibits an adenine-to-guanine transition at position 66, however no other polymorphisms separate these samples from reference genome Portsmouth (genotype I). Genotype III contains three SNPs relative to genotype I: the same adenine-to-guanine transition at position 66 seen in genotype II, an adenine-to-cytosine transversion at position 134, and a

guanine-to-cytosine transversion at position 236. This genotype includes the South American Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Ao240, Paty, and Ao10, as well as *R, parkeri* s. l. strains Parvitarsum Ch, Parvitarsum Ar, Black Gap, and tick lysate DP18-72. The fourth genotype for this locus includes only strain NOD Pantanal, with four SNPs relative to genotype I; this strain exhibits the three SNPs present in genotype III but also includes a guanine-to-adenine transition at position 52 relative to genotype I.

MC1_RS06595

There are five genotypes in this locus (Figure 4.4D). Genotype I for this locus consists of the 50 North American *R. parkeri* s. s. strains and tick lysates, all of which are identical to reference strain Portsmouth. Genotype II for this locus consists of the South American *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as the South American tick lysates AT-75, AT-137, AT-190, and AT-193. This genotype exhibits an adenine-to-guanine transition at position 229 relative to the reference genome Portsmouth (genotype I). The third genotype (genotype III) for this locus contains the South American Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Ao240, Paty, and Ao10 as well as *R. parkeri* s. l. strains Parvitarsum Ar and Parvitarsum Ch. This genotype contains the adenine-to-guanine transition at position 229 seen in the second genotype, but also has another adenine-to-guanine transition at position 53 relative to genotype I. The fourth genotype (genotype IV) is comprised of the North American strain Black Gap and tick lysate DP18-72 and contains the two adenine-to-guanine transitions at positions present in genotype III for this locus as well as a guanine-to-thymine transversion at position 121 relative to reference genome Portsmouth (genotype I). Strain NOD Pantanal is the only member of the fifth genotype (genotype V), with three SNPs relative to reference genome Portsmouth (genotype I): the same adenine-to-guanine transition at position

229 seen in the second genotype, as well as an adenine-to-guanine transition at position 116 and a guanine-to-adenine transition at position 97 relative to reference genome Portsmouth (genotype I).

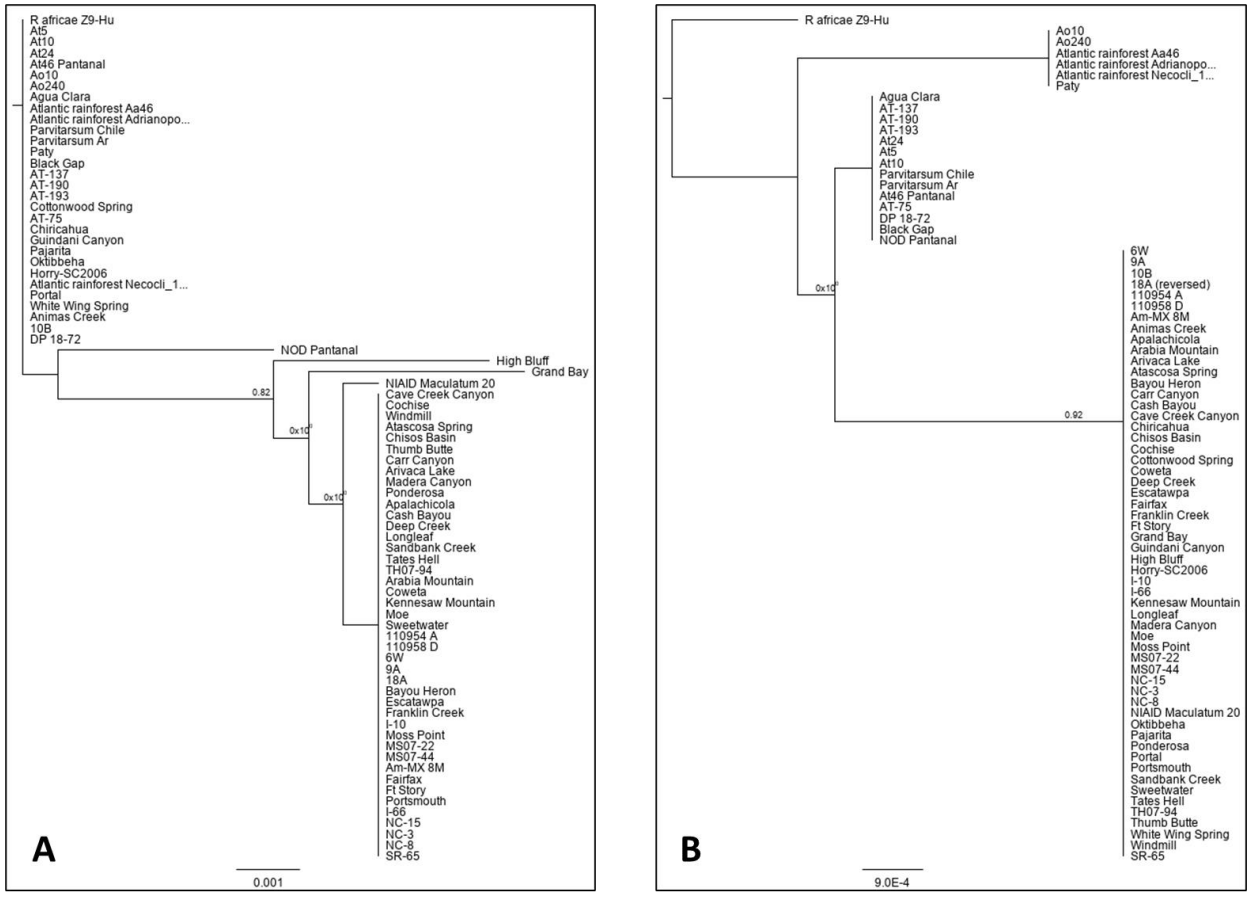

Figure 4.3 Individual phylogenies for coding loci

Phylogenies show **A)** the six genotypes for MC1_RS05545 **B)** the three genotypes for MC1_RS06275

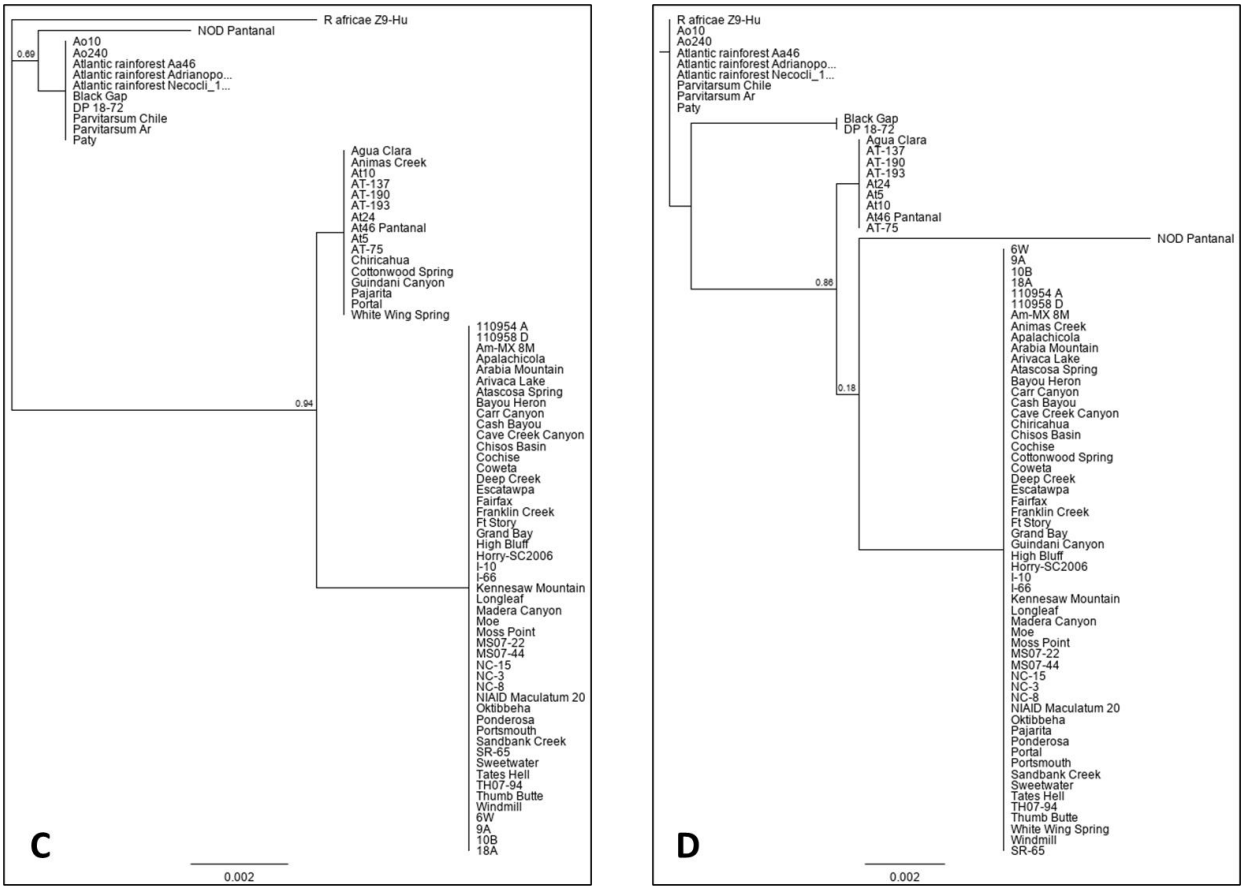

Figure 4.4 Individual phylogenies for coding loci

Phylogenies show **C)** the four genotypes of MC1_RS06395, and **D)** the five genotypes for MC1_RS06595.

Concatenated phylogeny

DNA sequences for all 77 rickettsial samples for the six loci were concatenated and aligned for phylogenetic analysis; the concatenated final alignment consists of 1,519 nucleotides. The loci were concatenated in the following order: MC1_RS05545, MC1_RS05545 - MC1_RS05550, MC1_RS06275, MC1_RS06595, MC1_RS03940 - MC1_RS03945, MC1_RS06395. The length of the amplicons for each individual locus is available in Table 4.2. After phylogenetic analysis, the *R. parkeri* s. s. and *R. parkeri* s. l. samples were well separated from both each other and *R. africae* Z9-Hu^T under high posterior probabilities (Figure 4.4).

The *R. parkeri* s. l. group was subdivided into three clades, all under robust posterior probabilities. These include the Parvitarsum/Black Gap clade (Clade 1), the Atlantic rainforest clade (Clade 2), and the NOD clade (Clade 3). Clade 1 includes *R. parkeri* s. l. strains Parvitarsum Ch and Parvitarsum Ar isolated from *A. parvitarsum* in Chile and Argentina, respectively. Strains Parvitarsum Ch and Parvitarsum Ar are completely clonal across all six loci. Also in this clade, separated from the Parvitarsum strains by strong posterior probability support, are strain Black Gap and tick lysate DP18-72, both from *D. parumapertus* in Texas and New Mexico, USA, respectively. Strain Black Gap and tick lysate DP18-72 are clonal at five of the six loci; however, tick lysate DP18-72 has a two base pair insertion as described above in intergenic region MC1_RS03940 - MC1_RS03945.

Clade 2 consists of the *R. parkeri* s. l. Atlantic rainforest strains Necocli_10_11 from *A. ovale* in Colombia, and Ao10, Ao240, Adrianópolis, and Paty from *A. ovale* in Brazil. Also in this clade is *R. parkeri* s. l. Atlantic rainforest strain Aa46, isolated from *A. aureolatum* in Brazil. There is clear separation between the Colombian strain Necocli_10_11 and the Brazilian strains, and strong support within this clade for the separation of Atlantic rainforest strains Paty and Aa46 from the rest of the group. Variation at each locus between the individual strains within this clade is described above.

Clade 3 contains the Brazilian isolate NOD Pantanal, the only strain in this study that was isolated from an *A. nodosum* tick. This clade has strong posterior probability support as a separate group from both the other two *R. parkeri* s. l. clades (Clades 1 and 2) and the *R. parkeri* s. s. samples.

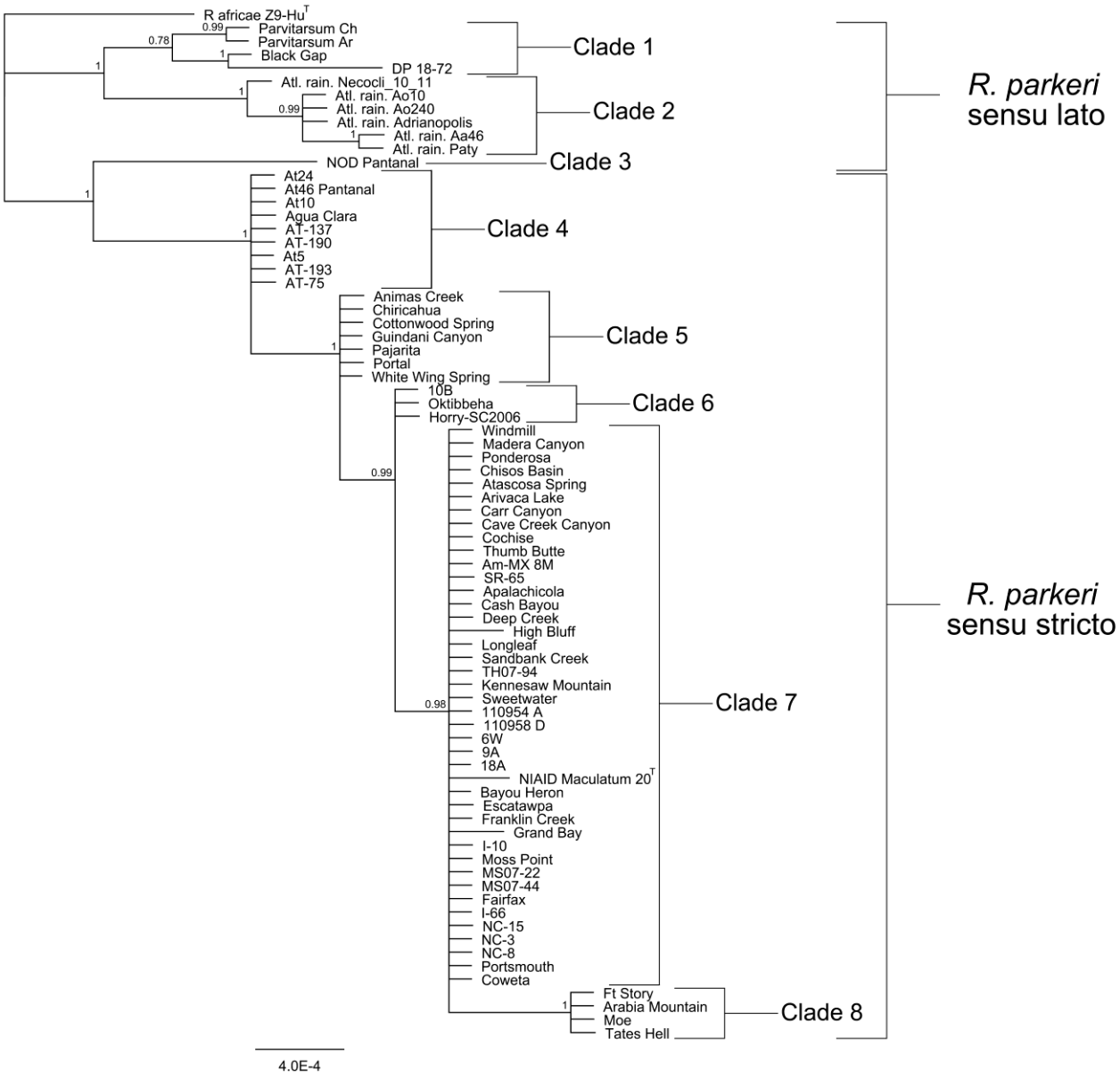

Figure 4.5 Concatenated phylogeny of *R. parkeri* s. s. and *R. parkeri* s. l.

Concatenated phylogenetic analysis of *Rickettsia parkeri* s. s. and *R. parkeri* s. l. strains using *Rickettsia africae* Z9-Hu^T as an outgroup. A total of 1,519 nucleotides representing 4 coding regions and 2 intergenic regions (Table 4.2) were concatenated and subjected to Bayesian analysis. Numbers at nodes are posterior probability support values, and clades are indicated by brackets and labeled.

The *R. parkeri* s. s. samples are divided into four clades. The first of these (Clade 4) consists of the South American *R. parkeri* s. s. isolates and tick lysates. All samples in this group

originated from *A. triste* in Brazil, Uruguay, or Argentina (Table 4.1), and the five isolates and four tick lysates from this clade are completely clonal at all six loci.

Clade 5 consists of seven isolates from *A. maculatum* s. l. in the southwestern United States: six from southern Arizona (Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring) and one from New Mexico (Animas Creek) (Table 41). There is robust posterior probability support to separate this group from both the South American *R. parkeri* s. s. samples (Clade 4) and the rest of the North American *R. parkeri* s. s. samples. The seven isolates in Clade 5 are identical to each other at all six loci analyzed and represent a mix of genotypes for the individual loci. For two of the four genes analyzed (MC1_RS05545 and MC1_RS06395), the isolates in this group are identical to the South American *R. parkeri* s. s. samples (Clade 4). For the other two genes and two intergenic regions, the seven isolates in Clade 5 are identical to the largest North American *R. parkeri* s. s. clade (Clade 7).

Clade 6 contains only three samples from the United States: *A. maculatum* s. s tick lysate 10B from Delaware, isolate Oktibbeha from *A. maculatum* s. s. in Mississippi, and isolate Horry-SC2006 from a human case in South Carolina (Table 4.1). These three samples are completely clonal and are separated from the largest North American *R. parkeri* s. s. clade (Clade 7) by one gene (MC1_RS05545), for which these three samples are identical to both Clades 4 and 5, representing the South American *R. parkeri* s. s. samples and a subset of the isolates from the Southwestern United States.

The largest clade for the *R. parkeri* s. s. samples is Clade 7, which contains 42 of the North American *R. parkeri* s. s. samples, including the reference genome strain Portsmouth and the type strain NIAID Maculatum 20^T (Table 4.1). Nearly all of the 42 samples in Clade 7 are clonal, however strains High Bluff and Grand Bay exhibit SNPs and NIAID Maculatum 20^T

exhibits a single base-pair insertion in gene MC1_RS05545, as described above. The final clade (Clade 8) nests within Clade 7 and consists of three U.S. isolates: strains Moe and Arabia Mountain from *A. maculatum* s. s. from Georgia, strain Tate's Hell from *A. maculatum* s. s. from Florida, and strain Ft. Story from a human case in Virginia. These four isolates share a deletion in gene MC1_RS03940 - MC1_RS03945 as described above.

Discussion

The work shown here corroborates recent analyses identifying clear separation between strains of *R. parkeri* s. s. and *R. parkeri* s. l. as well as distinct separation between North American and South American strains of *R. parkeri* s. s. (Nieri-Bastos et al. 2018). In addition, results of the current investigation reveal four unique North American clades of *R. parkeri* s. s. (Figure 4.4). Geographically, the most diversity within *R. parkeri* s. s. is seen in the eastern United States, where three separate clades are represented (Figure 4.4). This is likely due to the more extensive availability of suitable habitat for *A. maculatum* in this region relative to other areas where *R. parkeri* s. s. occurs in the United States. The long, humid summer season coupled with the wide availability of mammalian hosts in the Gulf Coast and eastern United States provides ideal conditions for this tick species and its pathogens to proliferate (Teel et al. 2010, Paddock and Goddard 2015). Found in these regions, the largest group of *R. parkeri* s. s. is Clade 7, which is also the most geographically expansive; the range of samples in this clade extends from northern Mexico to the state of Delaware (Figure 4.4), suggesting that this dominant strain represents the most widely dispersed and highly conserved strain in North America.

Figure 4.6 Map showing the distribution of *R. parkeri* in the Americas

Map showing all eight clades of *R. parkeri* s. s. and *R. parkeri* s. l. and their respective distributions in North and South America. Clade 1 is represented by black squares, Clade 2 by black triangles, Clade 3 with a white star, Clade 4 by black stars, Clade 5 by white triangles, Clade 6 by white circles, Clade 7 by black circles, and Clade 8 by white squares.

Conversely, Clade 5 contains seven samples restricted to the southwestern United States that appear to represent an intermediate genotype between the South American *R. parkeri* s. s. samples (Clade 4) and Clade 7 (Figure 4.4, Figure 4.5). The samples in Clade 5 were all isolated from *A. maculatum* s. l. ticks within the Madrean Occidental archipelago sky islands (Warshall 1995). These fractured riparian habitats are found in otherwise arid regions and likely depend on migratory birds, cattle, or other ungulates to disperse tick species to additional suitable habitats within the surrounding desert. There is no clear geographical or ecological distinction between the two genotypes from the southwestern United States; strains Pajarita (Clade 5) and Atascosa Spring (Clade 7) were collected on the same day in the Pajarito Mountains (Allerdice et al. 2017), the site of the first human cases in this state (Herrick et al. 2016), thus confirming that at least two genotypes are circulating together amongst *A. maculatum* s. l. tick populations.

Figure 4.7 North American distribution of *R. parkeri* s. s.

Clade 5: white triangles, Clade 6: white circles, Clade 7: black circles, Clade 8: white squares

While there is significant geographical separation between the *R. parkeri* s. s. samples in Clades 4 and 7, Clade 1 is the only group with cross continental representation, including *R. parkeri* s. l. strains from Chile, Argentina, and the United States (Figure 4.4). The origin of the close genetic relationship between samples from North American *Dermacentor parumapertus* and South American *Amblyomma parvitarsum* is not immediately clear; *A. parvitarsum* are ectoparasites of camelids in Argentina, Bolivia, Peru and Chile (Muñoz-Leal et al. 2014) while *D. parumapertus* are found on *Lepus californicus*, a common hare reported from the western United States and Mexico (Allred and Roscoe 1956, Paddock et al. 2017). However, the hosts of the immature stages of *D. parumapertus* could offer some insight. In addition to parasitizing *L. californicus*, immature *D. parumapertus* have been recovered from various small rodents, including several species whose ranges extend into central and eastern Mexico (Gastfriend 1955). A recent study of micromammals in Chile further identified *R. parkeri* s. l. strain Parvitarsum in ten different flea species, including those collected from *Rattus rattus*, a pest with cosmopolitan distribution (Moreno-Salas et al. 2020). To date, no immature *A. parvitarsum* have been collected from rodents; larval *A. parvitarsum* are ectoparasites of lizards, and the nymphal hosts are not known (Nava et al. 2017). Nonetheless, it is clear that rodents within this tick's range are likely infected with strain Parvitarsum, suggesting a potential route of entry for this bacterium into North American mammalian and tick populations through either infected rodents or infected fleas. It is important, however, to note the small sample size within this clade. Analysis of additional strains could perhaps increase the resolution between these two groups of rickettsial organisms and offer further insights into the origin of their close genetic relationship.

Although the novel typing scheme developed here identifies clear geographical distinction between strains of *R. parkeri* s. s. and *R. parkeri* s. l. for most of the groupings, this is not a completely consistent finding. While the two South American *R. parkeri* s. l. Parvitarsum strains clearly separate from the two North American *R. parkeri* s. l. Black Gap strains in Clade 1, the Parvitarsum isolates share 100% sequence identity, though they originate from Chile and Argentina. Similarly, in Clade 2, the single Colombian Atlantic rainforest strain (Necocli_10_11) separates from the Brazilian strains that comprise the rest of the clade; however, there is also strong support within this group for separation of the Brazilian Atlantic rainforest strains Paty and Aa46 from the rest of the clade (Figure 4.4). While the biogeography of these samples does not clearly align, the tick vector from which these strains are isolated could potentially inform their phylogenetic differentiation, echoing the results from previous work (Nieri-Bastos et al. 2018). In Clade 1, the *R. parkeri* s. l. strains can be separated based on their isolation from either *A. parvitarsum* (South American Parvitarsum strains) or *D. parumapertus* (North American Black Gap strains), and in Clade 2 strain Aa46 is the only *R. parkeri* s. l. Atlantic rainforest strain isolated from *A. aureolatum*. While Atlantic rainforest strain Paty from *A. ovale* also clusters with strain Aa46, Clade 2 has relatively few samples. It is possible that examining additional Atlantic rainforest strains from both *A. aureolatum* and other *Amblyomma* species in Brazil could elucidate the importance, if any, of the tick vector in relation to the rickettsial strain. Importantly, little is known about the reservoir hosts in nature for *R. parkeri*. It is possible that the genetic variation seen here could be partially attributed to habitat differences, and thus host differences, of the tick vectors from which the samples originated.

There have been confirmed human cases of *R. parkeri* rickettsiosis reported from Brazil, Colombia, Argentina, Uruguay, and the United States (Venzal et al. 2004, Nava et al. 2008,

Conti-Díaz et al. 2009, Romer et al. 2011, Portillo et al. 2013, Paddock and Goddard 2015, Herrick et al. 2016, Straily et al. 2016, Faccini-Martínez et al. 2018, Yaglom et al. 2020). In the United States, cases have been reported from 11 states: Arizona, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Texas, and Virginia (Herrick et al. 2016, Paddock and Goddard 2015, Yaglom et al. 2020). However, these states represent a small segment of the total distribution of *A. maculatum* in the country. Since the initial description of *A. maculatum* in 1844, its known range has expanded significantly, from that of a coastal tick with limited range of 100 – 200 miles inland in the Gulf Coast region to now being established in many central U.S. states such as Kansas, Oklahoma, and Illinois (Paddock and Goddard 2015, Phillips et al. 2020). Recent studies have confirmed the northern expansion of *A. maculatum* s. s. and *R. parkeri* s. s. into Connecticut (Molaei et al. 2021), representing the northernmost established populations of both tick and pathogen. This report by Molaei et al. highlights the continual expansion of *A. maculatum* s. s. into additional regions within North America and suggests an emerging public health risk for *R. parkeri* rickettsiosis in these areas.

While the typing scheme and resultant phylogenetic analyses presented here identify clear geographical separation between samples, the underlying causes for this separation are not immediately clear. Nonetheless, this work and that of others (Nieri-Bastos et al. 2018) reflects the increasingly recognized geographic range of *R. parkeri* in the Americas. This group of rickettsial organisms has been detected in 12 tick species from seven countries across two continents. It is reasonable to assume that these pathogens exist elsewhere in the Americas, as the recognized ranges of their tick vectors represent a more extensive distribution than the known range for *R. parkeri*. Future application of the multi-locus sequence typing analysis presented here could serve as a tool to help inform the origins or clarify the taxonomic status of novel *R.*

parkeri strains as they are discovered. Given the broad known distribution of *R. parkeri* s. s. and *R. parkeri* s. l. across North and South America and their associations with many ixodid tick vectors of medical importance, this group of emerging human pathogens already presents a public health threat to most of the Western Hemisphere.

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CHAPTER V

OVERALL DISCUSSION AND CONCLUSIONS

Results from this dissertation contribute valuable information towards understanding the systematics of the *Amblyomma maculatum* s. l. ticks found in southern Arizona and other regions of the southwestern United States and northern Mexico. Objectives for all individual experiments were met. One of the projects (Chapter II) has been published and another (Chapter IV) is in press. The studies completed as part of this dissertation examined relationships within *A. maculatum* as well as within *Rickettsia parkeri*. Additionally, the work presented here generated a novel typing scheme for *R. parkeri* and identified microsatellite loci for *A. maculatum* which can be used in future analyses with these organisms.

After the crossbreeding study, we now understand that there is likely reproductive incompatibility between *A. maculatum* s. l. from Arizona and *A. maculatum* s. s. from Georgia, suggesting that these two groups of ticks represent separate biological species. Because we did not perform any back crosses between the F1 hybrids to the homologous Arizona or Georgia ticks, we do not know if any viable F2 hybrids would have resulted from these tests. We thus do not know if some gene flow could still occur in a more natural situation between *A. maculatum* s. s. and *A. maculatum* s. l. through hybridizations and back crosses. This is important to note for populations that are largely allopatric but possibly share some sympatry. *Amblyomma maculatum* s. l. have been identified in Arizona, northern Mexico, New Mexico, and western Texas, while

the known southern range of *A. maculatum* s. s. extends from the Gulf Coast to central Texas. It is thus likely that at some point along the southern U.S. border, both *A. maculatum* s. s. and *A. maculatum* s. l. coexist. Studies on the reproductive compatibility of these two ticks in locations where they are sympatric would better elucidate whether the two popsulations are interbreeding, and if not, which pre- or post-zygotic reproductive isolating mechanisms are at work to keep them separate.

This crossbreeding analysis was the third species test for *A. maculatum* s. l.; the other two by Lado et al. were cited extensively throughout this work and consist of both molecular and morphological analyses. When looking at the results from all three of the species concepts tested for *A. maculatum* s. s. and *A. maculatum* s. l., there is still no clear and consistent delineation between the two populations. Molecular data suggest conspecificity, while both crossbreeding and morphological analyses resulted in the ability to separate them. The novel microsatellites developed as part of this work will hopefully be used to clarify these relationships by offering a broader understanding of the population genetics of both *A. maculatum* s. l. as well as the larger *A. maculatum* group.

The work performed and presented here with *Rickettsia parkeri* may also offer some insight into *A. maculatum* s. l. in the southwestern United States. Four genotypes of North American *R. parkeri* s. s. were identified through our multi-locus sequence typing (MLST) analysis. While three of these genotypes are broadly distributed across the United States, one genotype is exclusively found within the distribution of *A. maculatum* s. l. As collections continue throughout this distribution, the MLST designed here can be used alongside microsatellite analyses of *A. maculatum* s. l. to determine what degree of coevolution may be happening between tick and pathogen in this unique ecosystem.

The work presented here also highlights the growing importance of *R. parkeri* rickettsiosis and its vectors. As the range of *A. maculatum*, and thus *R. parkeri*, expands in the United States, cases are likely to increase and appear in new areas. This dissertation offers tools to analyze both pathogen and vector in those cases, thus creating a deeper understanding of the true public health risk for this emerging pathogen.

APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER II

Table A.1 Raw data from F0 feeding of GA ♂ / GA ♀ *A. maculatum* s. s

Rabbit #3: 17-03-03 (GA M/GA F) - Ticks placed 7/21/2017

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Date Oviposition Ended	Preoviposition Period (days)	Duration of oviposition (days)	eggs (g)
7/30/2017	A	4.654	5.517	0.863	9	8/5/2017	9/3/2017	6	29	0.467
7/30/2017	B	4.411	5.382	0.971	9	8/5/2017	9/3/2017	6	29	0.532
7/30/2017	C	4.399	5.293	0.894	9	8/5/2017	9/2/2017	6	28	0.545
7/31/2017	D	4.701	5.777	1.076	10	8/6/2017	9/3/2017	6	28	0.498
7/31/2017	E	4.498	5.613	1.115	10	8/5/2017	9/3/2017	5	29	0.6
7/31/2017	F	4.439	5.461	1.022	10	8/4/2017	8/31/2017	4	27	0.627
7/31/2017	G	4.479	5.086	0.607	10	8/6/2017	8/31/2017	6	25	0.346
7/31/2017	H	4.676	5.364	0.688	10	8/7/2017	8/31/2017	7	24	0.422
7/31/2017	I	4.637	5.627	0.99	10	8/5/2017	9/2/2017	5	28	0.576
7/31/2017	J	4.643	5.815	1.172	10	8/6/2017	8/31/2017	6	25	0.733
7/31/2017	K	4.719	5.945	1.226	10	8/6/2017	9/2/2017	6	27	0.749
7/31/2017	L	4.586	5.744	1.158	10	8/5/2017	9/2/2017	5	28	0.733
8/1/2017	M	4.552	5.179	0.627	11	8/8/2017	8/31/2017	7	23	0.388
8/1/2017	N	4.651	5.69	1.039	11	8/6/2017	8/31/2017	5	25	0.578
8/1/2017	O	4.664	5.403	0.739	11	8/3/2017	8/28/2017	2	25	0.442
8/1/2017	P	4.593	5.597	1.004	11	8/5/2017	9/2/2017	4	28	0.618
8/1/2017	Q	4.688	5.8	1.112	11	8/5/2017	9/2/2017	4	28	0.574
8/1/2017	R	4.511	5.576	1.065	11	8/4/2017	9/2/2017	3	29	0.465
8/1/2017	S	4.594	5.277	0.683	11	8/5/2017	8/31/2017	4	26	0.353
8/1/2017	T	4.659	5.89	1.231	11	8/6/2017	9/2/2017	5	27	0.822
8/2/2017	U	4.449	5.108	0.659	12	8/8/2017	8/31/2017	6	23	0.374
8/2/2017	V	4.583	5.732	1.149	12	8/7/2017	9/2/2017	5	26	0.733
8/2/2017	X	4.47	5.502	1.032	12	8/5/2017	9/2/2017	3	28	0.565
8/3/2017	Y	4.404	4.775	0.371	13	8/12/2017	9/2/2017	9	21	0.294

Table A.2 Raw data from F0 feeding of GA ♂ *A. maculatum* s. s. / AZ ♀ *A. maculatum* s. l

Rabbit #4: 17-03-06 (GA M/AZ F) - Ticks placed 7/21/2017

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Date Oviposition Ended	Preoviposition Period (days)	Duration of oviposition (days)	eggs (g)
7/28/2017	A	4.659	5.831	1.172	7	8/2/2017	8/31/2017	5	29	0.718
7/28/2017	B	4.59	5.039	0.449	7	8/2/2017	8/22/2017	5	20	0.178
7/28/2017	C	4.664	5.586	0.922	7	8/2/2017	8/24/2017	5	22	0.497
7/28/2017	D	4.612	5.573	0.961	7	8/3/2017	8/28/2017	6	25	0.718
7/28/2017	E	4.649	5.571	0.922	7	8/1/2017	8/28/2017	4	27	0.567
7/28/2017	F	4.415	5.181	0.766	7	8/2/2017	8/28/2017	5	26	0.424
7/29/2017	G	4.594	5.438	0.844	8	8/2/2017	8/28/2017	4	26	0.516
7/29/2017	H	4.431	5.452	1.021	8	8/3/2017	8/22/2017	5	19	0.652
7/29/2017	I	4.469	5.497	1.028	8	8/2/2017	8/28/2017	4	26	0.672
7/29/2017	J	4.551	5.63	1.079	8	8/2/2017	8/28/2017	4	26	0.702
7/29/2017	K	4.71	5.53	0.82	8	8/2/2017	8/26/2017	4	24	0.495
7/29/2017	L	4.622	5.455	0.833	8	8/3/2017	8/24/2017	5	21	0.544
7/29/2017	M	4.505	5.603	1.098	8	8/2/2017	8/28/2017	4	26	0.565
7/29/2017	N	4.662	5.325	0.663	8	8/1/2017	8/28/2017	3	27	0.401
7/29/2017	O	4.504	5.649	1.145	8	8/3/2017	8/28/2017	5	25	0.597
7/29/2017	P	4.658	5.465	0.807	8	8/2/2017	8/26/2017	4	24	0.451
7/29/2017	Q	4.672	5.466	0.794	8	8/2/2017	8/24/2017	4	22	0.446
7/30/2017	R	4.562	5.763	1.201	9	8/3/2017	8/31/2017	4	28	0.709
7/30/2017	S	4.44	4.819	0.379	9	8/5/2017	8/22/2017	6	17	0.118
7/30/2017	T	4.512	5.23	0.718	9	8/5/2017	8/28/2017	6	23	0.452
7/30/2017	U	4.687	5.224	0.537	9	8/4/2017	8/24/2017	5	20	0.347
7/31/2017	V	4.405	5.205	0.8	10	8/5/2017	8/22/2017	5	17	0.524
8/1/2017	W	4.717	5.331	0.614	11	8/4/2017	8/28/2017	3	24	0.449
8/2/2017	X	4.67	5.197	0.527	12	8/8/2017	8/28/2017	6	20	0.262

Table A.3 Raw data from F0 feeding of AZ ♂ / AZ ♀ *A. maculatum* s. l.

Rabbit #1: 17-03-04 (AZ M/AZ F) - Ticks placed 7/21/2017

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Date Oviposition Ended	Preoviposition Period (days)	Duration of oviposition (days)	eggs (g)
7/28/2017	A	4.422	5.309	0.887	7	8/3/2017	8/26/2017	6	23	0.557
7/28/2017	B	4.511	5.444	0.933	7	8/1/2017	8/26/2017	4	25	0.567
7/28/2017	C	4.487	5.161	0.674	7	8/3/2017	8/26/2017	6	23	0.409
7/28/2017	D	4.606	5.492	0.886	7	8/2/2017	8/28/2017	5	26	0.542
7/28/2017	E	4.544	5.439	0.895	7	8/3/2017	8/28/2017	6	25	0.534
7/29/2017	F	4.653	5.456	0.803	8	8/3/2017	8/17/2017	5	14	0.451
7/29/2017	G	4.582	5.313	0.731	8	8/3/2017	8/26/2017	5	23	0.437
7/29/2017	H	4.686	5.406	0.72	8	8/4/2017	8/25/2017	6	21	0.318
7/29/2017	I	4.521	5.317	0.796	8	8/3/2017	8/28/2017	5	25	0.469
7/29/2017	J	4.503	5.328	0.825	8	8/4/2017	8/25/2017	6	21	0.398
7/29/2017	K	4.662	5.698	1.036	8	8/3/2017	8/28/2017	5	25	0.538
7/29/2017	L	4.613	5.381	0.768	8	8/3/2017	8/19/2017	5	16	0.396
7/29/2017	M	4.748	5.772	1.024	8	8/2/2017	8/26/2017	4	24	0.59
7/29/2017	N	4.721	5.515	0.794	8	8/2/2017	8/22/2017	4	20	0.494
7/29/2017	O	4.707	5.686	0.979	8	8/2/2017	8/26/2017	4	24	0.623
7/29/2017	P	4.51	5.364	0.854	8	8/4/2017	8/26/2017	6	22	0.495
7/29/2017	Q	4.672	5.402	0.73	8	8/3/2017	9/3/2017	5	31	0.416
7/30/2017	R	4.559	5.465	0.906	9	8/2/2017	8/28/2017	3	26	0.591
7/30/2017	S	4.463	5.353	0.89	9	8/3/2017	8/26/2017	4	23	0.545
7/30/2017	T	4.442	5.138	0.696	9	8/4/2017	8/28/2017	5	24	0.4
7/31/2017	U	4.69	5.717	1.027	10	8/6/2017	9/3/2017	6	28	0.524
7/31/2017	V	4.497	5.652	1.155	10	8/4/2017	8/31/2017	4	27	0.719
7/31/2017	W	4.714	5.726	1.012	10	8/5/2017	8/31/2017	5	26	0.637
8/2/2017	X	4.67	5.197	0.527	12	8/8/2017	8/28/2017	6	20	0.262

Table A.4 Raw data from F0 feeding of AZ ♂ *A. maculatum* s. l./ GA ♀ *A. maculatum* s. s

Rabbit #2: 17-03-05 (AZ M/GA F) Ticks placed 7/21/2017

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Date Oviposition Ended	Preoviposition Period (days)	Duration of oviposition (days)	eggs (g)
7/30/2017	A	4.636	5.902	1.266	9	8/5/2017	8/31/2017	6	26	0.8
7/30/2017	B	4.576	6.104	1.528	9	8/5/2017	8/31/2017	6	26	0.949
7/30/2017	C	4.571	5.596	1.025	9	8/5/2017	8/31/2017	6	26	0.627
7/31/2017	D	4.508	5.438	0.93	10	8/6/2017	8/31/2017	6	25	0.554
7/31/2017	E	4.728	5.543	0.815	10	8/7/2017	8/22/2017	7	15	0.436
7/31/2017	F	4.53	5.569	1.039	10	8/6/2017	9/2/2017	6	27	0.745
7/31/2017	G	4.486	5.762	1.276	10	8/6/2017	9/2/2017	6	27	0.823
7/31/2017	H	4.711	5.624	0.913	10	8/7/2017	8/31/2017	7	24	0.539
7/31/2017	I	4.658	5.498	0.84	10	8/5/2017	8/31/2017	5	26	0.512
8/1/2017	J	4.678	5.096	0.418	11	8/9/2017	8/28/2017	8	19	0.168
8/1/2017	K	4.641	5.515	0.874	11	8/7/2017	8/31/2017	6	24	0.518
8/1/2017	L	4.387	5.199	0.812	11	8/8/2017	8/31/2017	7	23	0.448
8/1/2017	M	4.588	5.245	0.657	11	8/6/2017	8/31/2017	5	25	0.334
8/1/2017	N	4.482	5.009	0.527	11	8/7/2017	8/28/2017	6	21	0.125
8/1/2017	O	4.566	5.51	0.944	11	8/7/2017	9/2/2017	6	26	0.582
8/1/2017	P	4.564	5.561	0.997	11	8/8/2017	9/2/2017	7	25	0.593
8/1/2017	Q	4.54	5.242	0.702	11	8/7/2017	8/31/2017	6	24	0.426
8/1/2017	R	4.804	5.939	1.135	11	8/7/2017	9/2/2017	6	26	0.673
8/1/2017	S	4.679	5.634	0.955	11	8/8/2017	8/31/2017	7	23	0.481
8/1/2017	T	4.635	5.612	0.977	11	8/11/2017	9/3/2017	10	23	0.499
8/2/2017	U	4.536	5.642	1.106	12	8/12/2017	9/3/2017	10	22	0.626
8/3/2017	V	4.61	5.512	0.902	13	8/19/2017	9/4/2017	16	16	0.373
7/31/2017	W	4.714	5.726	1.012	10	8/5/2017	8/31/2017	5	26	0.637
8/2/2017	X	4.67	5.197	0.527	12	8/8/2017	8/28/2017	6	20	0.262

Table A.5 Raw data from F1 feeding of GA ♂ / GA ♀ *A. maculatum* s. s.

Rabbit #1: 17-12-07 (GA M/GA F) - Ticks placed 3/27/2018 - Previously fed on 170901

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Preoviposition Period (days)	eggs (g)
4/5/2018	A	3.677	4.606	0.929	9	4/11/2018	6	0.484
	B	3.76	4.588	0.828	9	4/12/2018	7	0.519
	C	3.787	4.931	1.144	9	4/13/2018	8	0.719
	D	3.676	4.838	1.162	9	4/12/2018	7	0.741
4/6/2018	E	3.757	4.731	0.974	10	4/13/2018	7	0.59
	F	3.686	4.875	1.189	10	4/12/2018	6	0.548
	G	3.781	4.913	1.132	10	4/12/2018	6	0.743
	H	3.685	4.663	0.978	10	4/12/2018	6	0.643
	I	3.757	4.728	0.971	10	4/13/2018	7	0.424
	J	3.686	4.727	1.041	10	4/13/2018	7	0.689
	K	3.783	4.656	0.873	10	4/13/2018	7	0.564
	L	3.784	4.17	0.386	10	4/13/2018	7	0.191
	M	3.684	4.932	1.248	10	4/13/2018	7	0.798
4/7/2018	N	3.76	5.206	1.446	11	4/14/2018	7	0.924
	O	3.783	4.635	0.852	11	4/13/2018	6	0.429
	P	3.816	5.088	1.272	11	4/13/2018	6	0.584
	Q	3.669	4.544	0.875	11	4/13/2018	6	0.55
	R	DAMAGED			11			
4/8/2018	S	4.362	4.885	0.523	12	4/16/2018	8	0.201
	T	4.551	5.715	1.164	12	4/15/2018	7	0.621
4/9/2018	U	3.749	4.549	0.8	13	4/16/2018	7	0.422

Table A.6 Raw data from F1 feeding of GA ♂ *A. maculatum* s. s. / AZ ♀ *A. maculatum* s. l.

Rabbit #1: 17-12-10 (GA M/AZ F) - Ticks placed 3/27/2018 - Previously fed on 170904

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Preoviposition Period (days)	eggs (g)
4/4/2018	A	3.668	4.918	1.25	8	4/10/2018	6	0.833
4/7/2018	B	3.812	4.957	1.145	11	4/13/2018	6	0.732
	C	3.75	5.107	1.357	11	4/13/2018	6	0.918
	D	3.669	4.855	1.186	11	4/12/2018	5	0.713
	E	3.784	4.909	1.125	11	4/10/2018	3	0.753
4/8/2018	F	3.756	4.541	0.785	12	4/14/2018	6	0.461
	G	3.79	5.043	1.253	12	4/14/2018	6	0.81
	H	3.758	4.68	0.922	12	4/14/2018	6	0.576
4/9/2018	I	3.761	4.911	1.15	13	4/14/2018	5	0.728
	J	3.752	4.894	1.142	13	4/16/2018	7	0.712
########	K	3.668	5.133	1.465	14	4/16/2018	6	0.893
	L	3.683	5.039	1.356	14	4/14/2018	4	0.847
	M	3.818	4.828	1.01	14	4/15/2018	5	0.269
	N	3.818	5.001	1.183	14	4/15/2018	5	0.756
########	O	3.79	4.88	1.09				
	P	3.816	4.423	0.607				0.239
	Q	3.784	4.115	0.331				0.101
	R	3.759	3.904	0.145				
	S	3.681	3.965	0.284				0.095
	T	3.816	3.874	0.058				

Cells in yellow indicate ticks that were partially engorged and attached when removed from the experimental animal.

Table A.7 Raw data from F1 feeding of AZ ♂ / AZ ♀ *A. maculatum* s. l.

Rabbit #1: 17-12-08 (AZ M/AZ F) - Ticks placed 3/27/2018 - Previously fed on 170902

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Preoviposition Period (days)	eggs (g)
4/4/2018	A	3.751	4.387	0.636	8	4/10/2018	6	0.427
4/5/2018	B	3.75	4.446	0.696	9	4/11/2018	6	0.46
	C	3.67	4.352	0.682	9	4/10/2018	5	0.426
	D	3.671	4.761	1.09	9	4/10/2018	5	0.724
	E	3.672	4.593	0.921	9	4/11/2018	6	0.633
	F	3.751	4.621	0.87	9	4/11/2018	6	0.559
	G	3.751	4.728	0.977	9	4/11/2018	6	0.673
	H	3.679	4.482	0.803	9	4/10/2018	5	0.498
4/6/2018	I	3.761	4.57	0.809	10	4/12/2018	6	0.536
	J	3.75	4.595	0.845	10	4/11/2018	5	0.564
	K	3.812	4.999	1.187	10	4/11/2018	5	0.768
	L	3.783	4.801	1.018	10	4/10/2018	4	0.68
	M	3.758	4.369	0.611	10	4/12/2018	6	0.398
4/7/2018	N	3.672	4.693	1.021	11			
	O	3.758	4.294	0.536	11	4/12/2018	5	0.306
	P	3.751	4.669	0.918	11	4/11/2018	4	0.58
4/8/2018	Q	3.681	4.668	0.987	12	4/13/2018	5	0.671
	R	3.667	4.608	0.941	12	4/13/2018	5	0.648
	S	3.668	4.427	0.759	12	4/13/2018	5	0.229
	T	3.682	4.747	1.065	12	4/12/2018	4	0.727

Table A.8 Raw data from F1 feeding of AZ ♂ *A. maculatum* s. l./ GA ♀ *A. maculatum* s. s.

Rabbit #1: 17-12-09 (AZ M/GA F) - Ticks placed 3/27/2018 - Previously fed on 170903

Date Removed	Identifier	Tube (g)	Tube + F (g)	F (g)	Feeding Duration (days)	Date oviposition started	Preoviposition Period (days)	eggs (g)
4/8/2018	A	4.488	5.086	0.598	12	4/13/2018	5	
4/9/2018	B	3.684	4.334	0.65	13	4/15/2018	6	
4/11/2018	C	3.757	4.157	0.4				
	D	3.751	3.961	0.21				
	E	3.681	3.92	0.239				
	F	3.682	3.797	0.115				
	G	3.756	4.143	0.387				
	H	3.758	3.95	0.192				
	I	3.781	4.198	0.417				
	J	3.76	4.05	0.29				
	K	3.783	4.009	0.226				
	L	3.758	3.955	0.197				
	M	3.669	3.888	0.219				
	N	3.75	3.949	0.199				
	O	3.76	4.115	0.355				
	P	3.759	3.97	0.211				
	Q	3.784	4.153	0.369				
	R	3.752	4.187	0.435				
	S	3.758	4.033	0.275				
	T	3.682	4.747	1.065	12	4/12/2018	4	0.727

Cells in yellow indicate ticks that were partially engorged and attached when removed from the experimental animal. The egg mass weight was not recorded.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER IV

Table B.1 Primers tested for this study.

Locus Tag or Title	Primer F	Tm (℃)	Primer R	Tm (℃)	Amplicon Size
Primers designed for this study					
MC1_RS03940 - MC1_RS03945	**AGG TGT ATA CAT AAA AGT CTC CA**	**54.5**	**CTT ATC TCT CGC ACC TTG GT**	**54.5**	**191**
MC1_RS05545 - MC1_RS05550	**GTG CAG TTC TGT TGT CAT CC**	**57**	**TGC TTG AAT GTA CCG GAG AA**	**56.9**	**213**
MC1_RS05545	**CTG GAT CCC GTG GTC AAG TC**	**60.1**	**AGA TGC CGA GCT TGG TAG AG**	**59.3**	**279-295**
MC1_RS06275	**TAA CAC CGC CTG CAA GCT TA**	**60**	**GTT ATA GGC GGT GGT CCA GG**	**59.9**	**271**
MC1_RS06395	**TTA TTA CCC GTG CCG GTT CC**	**60.1**	**TCT CCA CTC CTT CCG GTT CT**	**59.9**	**322**
MC1_RS06595	**GGG GCT AGT AAA CGG TGG TG**	**60.4**	**AAT ATT GTA AGC CCG CCG CC**	**61.2**	**375**
sca2	GTT GCA TAC CCT GTA TCG TC	56	TCC TAG AAC TGA AAT AGG GCA (RP) TGG TCC TAG AAC TGA AAT AGG (BG AR)	55.8 54.1	475-535
MC1_RS06410	TAT TAC CAC CGG TAG CCC CA	60	CCG ATG GGA TAG GAG CTC CT	60.3	295
rickA	GCC CTG CTA GTT CTC TCA TT (RP) GGC CCT GCT ATT TCT CTC ATT (AR) TGG CCC TAC TAT TTC TCT CAT T (BG)	57 57.8 56.3	GGT TGC TGA AAT TAG GGC AG	57.1	736
MC1_RS06625	AGC CCA CAA ATC GAA GGG TT	59.9	GCT AAC GGC AAG ACA AGCA G	59.8	306
MC1_RS06780	TGC AGG CGT TAC TTT CCA CA (RP) TGC TGG CGT TAC TTT CCA CA (AR)	60.2 60.2	CGA CGA TGG CTA AAC AGG CT	60.5	191
MC1_RS08360	TAG CCC CAT CGC TCT TAG GA	59.8	TGG ATC AGG TCA ACC TCA CG	59.4	236
Primers from Karpathy et al. 2007					
spo0J-abcT1-F	AAA GAT TTG GAA GAA TTA GAC TTG AT		TTT GCT TAA ACC AAC CAT TTC A		344
RR0155-rpmB-F	TTT CTA GCA GCG GTT GTT TTA TCC		TTA GCC CAT GTT GAC AGG TTT ACT		294
RR0345-tolC-F	AGA AGC TTC CGG ATG TAA TA		AGC AAA TAA AAA CCC TAA TAA C		237
cspA-ksgA-F	CAT CAC TGC TTC GCT TAT TTT		ATT TCT TTT CTT CCT CTT CAT CAA		403
RR1372-RR1373-F	TCC CGC GCC AGT ATC CA		CGG CGG CCA AAA TGC TA		347
RR1240-tlc5b-F	CGG GAT AAC GCC GAG TAA TA		ATG CCG CTC TGA ATT TGT TT		359

Table B.1 (continued)

tRNAPhe-nifR3-F	TTG AAC CAA CGA CAC AAG GA	CCG TAA CAC CTG ACA TTG GA	398
RC0098-dcd-F	CCG ATG CAA GGC AAA TAA TA	CGC AAA GGG CCT TAT CAT AC	398
RC0102-RC0103-F	GCG ATA AGC GAT TTA TTA GGC	GAA AGC CTA AAG CCT CCA CA	307
RC0280-23SrRNA-F	CAA AAA GCC GAC AAA GCC TA	CCT TCA TCG CCT TCT AGT GC	350
acrD-hupA-F	GGG CGT TTA ATA CAA ATT TTA GAC A	CAA TTC TCC TTT GAT AGG TTA ATA TGT	412
pal-RC1201-F	TGC AAG CAC ACA TAA TGC AA	TCA AAA TCG ATT CCT CTT TTC C	288
secB-czcR-F	ATG CAG GAT TCC AGC CTT TA	GGC TCG CCT TCA ATT AAC AA	335
groES-RC0970-F	CTT GCA TCG GCT TTT CTT TT	AGC TTT GAG CTG ATG GGC TA	352
secA-prsA-F	GCA GGT TCA AGC GAG TTA ATT T	AAA AGC AAT ACC GGA AAG CA	369
RC0604-RC0605-F	AAA GGC AAT AAC GGC AAA AA	AGC TCG CCA GTT CAT TCA TC	409
RC0409-trmU-F	AAC CTT GAC GTG CAT ATT CTA AA	GCC TGA CAT TGC GAC AAC TA	401
yqiX-gatB-F	CTG CGG CAG TAC CGA CTA TT	ATC CGA CGC TTG TGA ATC AG	396
rne-coxW-F	CGG AAA AGA ATG CAG AGT CTT G	CCA TTT TGT AAT TAA ACT TTT CTG	294
dnaN-RC0584-F	TCG TCA TGC CTG TTA AGG TG	TTG GAT AAT CAC CCG CTA AGA	461
lig-tgt-F	TTT TTG TGC TTC CTC TTC AGA T	CCA AAA TCT CAT GAG CCG TA	393
rho-RC0760c-F	CGG TAT TGT TAA GTT CTG CTG TG	TGC ATG CCA TTA CTT ATT ACA AAT G	483
folC-bioY-F	AGG TCG GCA CCG GAA AAT	TAC GGC GGC GTA TTA CCT T	375
ntrY-rpsU-F	AGC TGC TGT TGC TAA AGT AAA AA	CAA GAA GCA GCA AGA AGA CAG A	499
dksA-xerC-F	TCC CAT AGG TAA TTT AGG TGT TTC	TAC TAC CGC ATA TCC AAT TAA AAA	224
murG-RC0563-F	GAA GAA AAG AAG GGC ATA AGC TA	CAA GCT GAA AGT AAA AAC ATT CC	499
fabZ-lpxD-F	TGT TAG GAT CGA TTT TAA GTA CTC TAT CT	TGG ATT GGC ATA GAC AAT CTA TTA	356
rrf-pyrH-F	GAG CTT TCT CCA TCT TTT CTT G	AAA GGG GAA TAT ACG ACA ATT GAG	366
tRNAGly-tRNATyr-F	AGC TTG GAA GGC TGG AAC TC	ATC CTT CTC CCT CCA CCA CT	192
nusG-rplK-F	CAG TTG CAA TAT TGG TAA AGC A	CAG CAG CTG GAA TTA TCA AGT T	390
pcnB–sca1-1d-F	GCT CCC GCG GCA CTT AGA	TGC AAA TCA TAT GGC GGT AGG	247
pcnB–sca1-2d-F	TCA TGG TAA AAG GCA GAG ATA A	AAG GCA TTT TTG GAG CAG T	200
pcnB–sca1-3d-F	AAT TTC GGC TTT CTC ACA	CTT GGC GTT TGC TTG GTC T	290

The top section are primers designed in this study and the bottom section is primers previously published in Karpathy et al. 2007. Red letters in the primer sequences indicate modifications made to ensure the primers would bind to *R. parkeri*. The bolded entries in the top section indicate loci selected and used in this analysis. BG: Black Gap, AR: Atlantic rainforest, RP: *R. parkeri* s.