Spring 2021

Determination of the Presence of *Rickettsia* Spp. and *Borrelia* Spp. Carried by Tortoise Ticks from Madagascar

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DETERMINATION OF THE PRESENCE OF *RICKETTSIA* SPP. AND *BORRELIA* SPP. CARRIED BY TORTOISE TICKS FROM MADAGASCAR

by

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B.S. May 2015, Old Dominion University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

BIOLOGY

OLD DOMINION UNIVERSITY
May 2021

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ABSTRACT

DETERMINATION OF THE PRESENCE OF *RICKETTSIA* SPP. AND *BORRELIA* SPP. CARRIED BY TORTOISE TICKS FROM MADAGASCAR

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Ticks were removed from three species of Malagasy tortoises, *Astrochelys yniphora, A. radiata,* and *Pyxis arachnoides* (comprising two subspecies *P. a. arachnoides* and *P. a. oblonga*), between 2012 and 2015. The ticks were presumed to be from the genus *Amblyomma*. Ticks were morphologically identified and then checked molecularly to confirm their classification or identify any ticks that could not be morphologically identified. Molecular identification was done via end-point PCR that amplified tick cytochrome oxidase (CO1) and tick 12S rRNA genes. Ticks were screened via a real-time polymerase chain reaction assay for the presence of *Rickettsia* spp*.* and *Borrelia* spp*.,* amplifying the rickettsial 17 kDA and *Borrelia* 16S rRNA gene, respectively. Those positive for either pathogen were analyzed to determine the specific species via end-point PCR and sequencing. One hundred eighty-three ticks out of 239 tested ticks (77%) were positive for the presence of *Rickettsia* spp. and/or *Borrelia* spp.; *Rickettsia aeschlimannii* and *Rickettsia africae* were sequenced from *Rickettsia*-positive ticks and a *Borrelia* species related to *Borrelia turcica* was sequenced from the *Borrelia*-positive ticks. The aim of this study was to determine the presence of *Rickettsia* spp*.* and *Borrelia* spp*.* infecting the ticks, as well as identifying the ticks, in order to determine veterinary and public health risks. This will help further our understanding of these ticks and their pathogens and their relationship to these tortoises, as well as the impact they may have on both human and veterinary health.

ACKNOWLEDGEMENTS

The completion of this thesis would have not been possible without the help and contribution of those who guided me. I extend my sincerest thanks to my committee members for their patience and the time they took for my research and manuscript edits. I would also like to give a special recognition to my major advisor for all the time and effort spent helping me.

TABLE OF CONTENTS

Page

LIST OF TABLES vi

LIST OF FIGURES vii

INTRODUCTION 1
HOST-PARASITE DYNAMICS 1
PARASITE-REPTILE INTERACTIONS 3
TICK-REPTILE INTERACTIONS 4
TICK-TORTOISE INTERACTIONS 6
TICKS OF MALAGASY TORTOISES 6
AFRICAN TICK-BORNE PATHOGENS 7
MALAGASY TORTOISES 9

METHODS 11
TICK COLLECTION 11
DNA EXTRACTION 11
MOLECULAR TICK IDENTIFICATION 12
RICKETTSIA SPP. TESTING AND SEQUENCING 14
BORRELIA SPP. TESTING AND SEQUENCING 15
SEQUENCE CURATION AND PHYLOGENY ANALYSIS 16

RESULTS 18
TICK INFESTATION OF TORTOISES 18
BORRELIA AND *RICKETTSIA* SCREENING 18
BORRELIA GENETIC DIVERSITY 19
TICK IDENTIFICATION 25
TICK-PATHOGEN-TORTOISE RELATIONSHIP 28

DISCUSSION 29

CONCLUSION 34

REFERENCES 36

APPENDIX 43

VITA 50

LIST OF TABLES

Table Page

1. Primers and probes used to determine tick species 13

2. Primers and probes used to identify *Rickettsia* spp. 15

3. Primers and probes used to identify *Borrelia* spp. 17

4. Degree of similarity between the 12S DNA sequences of 12 samples 26

LIST OF FIGURES

Figure Page

1. Heat map of the northern Madagascar tortoise sampling locations 12

2. Heat map of the northern Madagascar tortoise sampling locations 13

3. Number of ticks positive for *Borrelia* spp. and/or *Rickettsia* spp. out of a total of 239 ticks..... 18

4. Phylogenetic analysis based on the concatenation of *flaB*, *uvrA*, and *rplB* of the genus *Borrelia* 20

5. Phylogenetic analysis based on the concatenation of *flaB* and *uvrA* of the genus *Borrelia* 21

6. Phylogenetic analysis based on the amplification of *flaB* of the genus *Borrelia* 22

7. Phylogenetic analysis based on the amplification of *uvrA* of the genus *Borrelia*..... 23

8. Phylogenetic analysis based on the amplification of *rplB* of the genus *Borrelia*..... 24

9. Phylogenetic analysis based on tick 12srDNA..... 27

INTRODUCTION

Unlike other taxa such as birds and mammals, which are typically valued for food resources and aesthetics, reptiles are less valued and understudied (Czech et al., 1998) despite being a species rich group that greatly contributes to biomass and has various roles in ecosystems. Many reptiles are at an increased risk of extinction due in part to an increase in disease-induced declines, resulting from a growing risk of emerging infectious diseases that have occurred over the past 20 years (Bower et al., 2019).

Parasites are also a taxonomically diverse group of organisms whose behaviors and effects on hosts differ based on their host species and environment. However, they are physically dependent on other organisms, which may be detrimental or beneficial to their host depending on the parasite species (Bower et al., 2019). Parasites of reptiles are often neglected, as most reptile studies focus on the ecology and conservation of the reptile host, rather than a zoonotic potential of the parasite. However, an increase in emerging infectious diseases signifies a need for a deeper understanding of these host-parasite interactions (Bower et al., 2017).

Host-Parasite Dynamics

Host-parasite dynamics are often influenced by environmental factors, such as temperature and climate, habitat changes, and anthropogenic effects. Sudden changes in environmental factors can result in stress-induced physiological changes in animals, resulting in a trade-off of energy between different biological functions. Reptiles, as ectotherms, are disproportionately affected by their external environment. Suboptimal conditions, such as cold temperatures, often force a re-allocation of energy to physiological processes such as thermoregulation from other systems such as the immune system, which in turn may lead to immunosuppression. For example, Kemp's ridley sea turtles (*Lepidochelys kempii*) that were cold-stunned had an increased number of bacterial and fungal infections, suspected to be the result of opportunistic infections (Innis et al., 2009). The immune systems of reptiles are dependent on ambient temperature and environmental conditions, and changes resulting in them living in suboptimal conditions can put the animals at increased risk for infection. Anthropogenic effects, such as the introduction of contaminants and habitat loss, can also cause dramatic environmental changes

that influence the reptiles' immune system. An increase in mercury concentrations in the Loggerhead sea turtles' (*Caretta caretta*) natural habitat was correlated with an increase in viral infections, indicating that heavy metals, introduced into the ocean through human activity, was causing immunosuppression in the turtles (Day et al., 2007). Human encroachment into a habitat can also affect host-parasite dynamics, especially when habitat area becomes smaller. Such habitat changes often affect both host and parasite prevalence in an area, as the density of host individuals can determine parasite load (Mugabo et al., 2015). Higher host population densities often result in competition for food and increased stress, which may reduce immunity within the population (Bower et al., 2019), as seen when high densities of the lizard *Zootoca vivipara* were observed to have inflammation and increased *Ixodes ricinus* tick infestations (Mugabo et al., 2015).

Parasite burdens can place physiological stress on reptiles. Maintaining an effective immune response against parasites is costly and often competes with other functions such as growth (Mugabo et al., 2015); disruptions in other physiological functions can influence fitness and interactions both within and between species (Bower et al., 2019). One of the more serious effects is an increased risk of predation. Certain parasites, typically endoparasites, can cause abnormal cellular growth, causing physical abnormalities and/or muscle damage (Bower et al., 2019). Such physical abnormalities not only negatively influence regular functions, but they can also reduce an individual's ability to escape predators. Physiological costs from parasites may also reduce the competitive ability between species; Schall (1992) showed an example of parasite-mediated competition in *Anolis* lizards of the Caribbean islands that determined the distribution of different *Anolis* populations. There are some cases where an individuals' own natural processes may cause immunosuppression, putting them at an elevated risk for parasite infection. Olsson et al. (2000) found testosterone-treated male sand lizards (*Lacerta agilis*) had a higher tick burden than those with lower testosterone levels. The authors suggested that immune suppression was associated with elevated testosterone levels due to balancing the energy costs for both reproductive success and maintaining immune function.

However, research is now shifting to analyzing host social networks to observe parasite abundance and distribution throughout host populations. Godfrey et al. (2006) observed that parasites with lower mobility, such as ticks, rely on the spatial proximity of hosts for transmission, suggesting that interactions between individuals can strongly affect parasite transmission. Parasites that have direct transmission routes of infection depend more on intra-group interactions rather than inter-group

interactions, while parasites carried by vectors are less dependent on social contact among hosts. Populations of the tuatara (*Sphenodon punctatus*) have increased tick infestations due to the social interactions among individuals. Studies on the tuatara have shown that male biased parasitism is common, most likely due to males overlapping with other individuals in competition for food and territory unlike females (Godfrey et al., 2010).

While host population density plays a large role in parasite abundance and distribution, host population genetic diversity can also influence parasite loads and disease dynamics (Bower et al., 2010). Studies have shown that host genetic variation dictate parasite susceptibility, with homogeneous populations often suffering from increased susceptibility, while genetically diverse populations were less susceptible to infection (Whiteman et al., 2006; Altermatt and Ebert, 2008). Increased susceptibility to parasites and/or disease correlates with a loss of genetic diversity, especially in inbred populations, resulting in the accumulation of potential deleterious mutations and/or the loss of resistance genes (Whiteman et al., 2006). One aspect of host genetic adaptation to pathogens is the Major Histocompatibility Complex (MHC) expression. The MHC is a highly polymorphic component of the immune system that allows multiple responses to different infections. Radwan et al. (2014) found a positive correlation between tick load on Ornate dragons (*Ctenophorus ornatus*) and MHC diversity, which agrees with the hypothesis that MHC polymorphism is driven by selective pressure associated with a given parasite (Spurgin and Richardson, 2010). However, more studies need to be done on population MHC diversity and parasite abundance as the relationship between MHC polymorphism and parasite load in reptiles is still unclear (Bower et al., 2019). Individual host responses to infection also differ at the gene expression level. Some infections cause species-specific differences in gene expression which can in turn dictate parasite evolutionary trajectory. More studies on genetic variation of both hosts and parasites influence rapid parasite evolution will contribute to a greater understanding of disease dynamics (Bower et al., 2019).

Parasite-Reptile Interactions

Parasites, ranging from viruses and bacteria to larger eukaryotic organisms, often exploit their hosts for survival, resulting in a variety of outcomes to host population (Bower et al., 2019). While eukaryotic parasites can play a role in maintaining reptile populations, when combined with other stressors, parasites can lead to major declines in many reptile species. Parasitic infections can affect population viability and increase susceptibility to other stressors, which puts reptiles at a risk of decreased reproduction and growth. This risk is especially important for rare and endangered species, such as the

Malagasy tortoises *Astrochelys* spp. and *Pyxis* sspp. While the acute stress response can aid animals in finding refuge for growth and/or recovery (Currylow et al., 2017a), chronic stress, especially in critically endangered species can be deleterious. Changes in environmental conditions, from both natural climate shifts and translocation from conservation efforts, can increase concentrations of the stress hormone corticosterone in *A. yniphora* and prevent breeding success for several years while the tortoises acclimatize to the changes (Currylow et al., 2017b). Flattened musk turtle (*Sternotherus depressus*) populations have also been experiencing major population declines due to environmental change and poaching. Such stress has led to a weakened immune system and consequently a higher risk for disease (Bower et al., 2019). Even some conservation strategies for endangered reptiles can lead to an increased risk of parasite spread.

The movement of reptiles in both wildlife reintroduction/translocation programs, as well as the exotic pet trades may inadvertently relocate parasites, which in turn can lead to parasitization of susceptible hosts with no immune response towards that particular parasite (Bower et al., 2019). The exotic reptile trade is often responsible for the introduction and spread of exotic parasites, as shown in Italy where the release of exotic pet tortoises was linked to an increased risk of helminth infection in native species (Cervone et al., 2016). In Florida, native snake species were shown to be more competent hosts of the parasite *Raillietiella orientalis* than the invasive Burmese python (*Python bivittatus*) host that introduced it to the area. This facilitated the parasite's spread beyond the Burmese python's range (Miller et al., 2020). Conservation of reptiles is an important goal associated with parasite-reptile studies; more knowledge on how hosts and parasites interact with each other and how anthropogenic impacts affect such interactions is required.

Tick-Reptile Interactions

Ticks are ectoparasites and are well-known vectors of various pathogens. As vectors of many human and veterinary pathogens, second only to mosquitoes worldwide, ticks are often the focus of potential public health and veterinary implications that may arise from the pathogens they carry (Sonenshine et al., 2002). While ticks and their pathogens are an important consideration in veterinary health, there is little information on the relationships between ticks and reptile hosts (Burridge, 2001).

The ecology of host taxa can determine the ecology of sympatric tick species. Reptiles can undergo pronounced niche shifts, such as ecological divergence, seasonal activity patterns, social interactions,

and physiological changes such as sexual dimorphism as they mature (Shine and Wall, 2004); these shifts can influence the intensity and prevalence of tick parasitism (Natusch et al., 2018). For example, Natusch et al. (2018) found that larger snakes were positively correlated with heavy tick loads. The broader scale pattern and loose overlap of larger snakes provided an easier attachment surface for ticks. Larger snakes also tended to have larger refuge sites that overlap with other snakes, allowing for more interactions with other snakes and increasing the chance of contact with ticks. This supports similar observations in other reptiles; reptiles using the same leaf litter can increase an animal's tick burden, as each individual is making frequent contact with multiple hosts infested by various tick life stages (Natusch et al., 2018). In some reptile species, females often have higher tick burdens than males, which may be due to lower immunocompetence resulting from a physiological trade-off for reproduction (Poulin, 2011). In other reptile species, the males have higher tick burdens as they have increased movement in an area as they look for mates and, in doing so, come into contact with ticks more frequently (Aubret et al., 2005). Ticks can also indirectly affect their own ecology depending on any alteration they cause to their host's behavior. While this topic requires more research, males of some reptile species with tick infestations show reduced aggression and less success in territorial contests (Godfrey et al., 2010). This can influence the reptile host's contact with other individuals and thus reduce transmission of ticks to new hosts.

Reptiles have also been implicated as potential reservoirs in tick-borne pathogen cycles by maintaining pathogen endemicity in reptile habitats (Cervone et al., 2016). As a diverse taxonomic group, reptiles may play a role in the epidemiology of various tick-borne pathogens. A number of different *Borrelia, Rickettsia, Ehrlichia, Anaplasma,* and *Babesia* species have been reported in lizards and other reptiles. *Ehrlichia ruminantium*, a livestock pathogen, has been found in *Amblyomma sparsum* ticks feeding on tortoises, and *Ehrlichia canis* was identified in *Amblyomma latum* ticks feeding on monitor lizards (*Varanus niloticus*) (Omondi et al., 2017). In contrast to *Borrelia* species such as *Borrelia burgdorferi*, other tick-borne pathogens are poorly studied in reptiles (Václav et al., 2010), in part because *Borrelia* species are seen as a greater threat to public health.

Overall, there is little data available on the impact of reptile ticks on their hosts; although there is some evidence that heavy tick loads can be detrimental to reptile health (Dunlap and Mathies, 1993). A heavy tick infestation can reduce hemoglobin concentrations in blood, which prevents effective oxygen transfer to the tissues (Bower et al., 2019). Some tick infestations may also cause respiratory distress, or

even death, if the ticks physically block nasal passages (Burridge, 2001). Ticks may also be vectors of detrimental reptile pathogens. For example, *A. latum* commonly infests snakes in Africa where it is a vector for reptile protozoal and bacterial pathogens. *Amblyomma latum* is also frequently reported outside its natural home range as a result of anthropological factors (Vercone et al., 2016); this species may then bring with it an increased risk of infection to native reptile species that may be detrimental to such hosts.

Tick-Tortoise Interactions

Tortoises are of interest in of zoonotic disease transmission because they are typically long lived and therefore may play a role in long-term maintenance of infectious agents (Pastiu et al., 2012). Tick-burdened tortoises often wander near humans and livestock, increasing the potential of pathogen spillover. *Amblyomma* species are common ticks of tortoises; they are notoriously aggressive and have been observed to actively pursue hosts. *Amblyomma hebraeum* and *Amblyomma variegatum*, two common tortoise ticks in sub-Saharan Africa, can cause issues with domestic livestock. They readily feed on wild ungulates such as giraffes and wildebeest while maintaining pathogens in the environment that can spill over into human society (Jensenius et al., 2003). Juvenile stages of these ticks may be less host-specific than adults, which could make them important in maintaining pathogens in the environment, as they may infest smaller animals such as birds, lizards, and small mammals (Pastiu et al., 2012).

The international trade of tortoises in the exotic pet trade has played a role in the epidemiology of tick-borne pathogens originally endemic to Africa. Many tortoises infested with ticks have been introduced to new locations, which can result in the exotic ticks establishing populations in new areas. In 1997, *Amblyomma marmorerum*, an African tortoise tick, was found to have become established in Florida near a reptile breeding facility. This particular tick can feed on a variety of reptiles, such as snakes and monitors, which threatens those reptile hosts that do not have resistance to novel pathogens carried by these ticks (Burridge, 2001). Like other reptiles, the interactions between ticks and tortoises are not well studied, and more research is needed to determine any pathogenic effects on the tortoises.

Ticks of Malagasy Tortoises

Very little is known about exotic reptile tick biology other than identification of host species and source geographic information. Knowledge on the pathogenic effects on hosts and vector potential is lacking.

Nine out of 29 ticks exotic to the US are known to be associated with disease (Burridge and Simmons, 2003).

Amblyomma chabaudi, a tortoise tick found in Madagascar, is not as well studied as other reptile ticks. The preferred host species of *A. chabaudi* is the spider tortoise (*Pixis arachnoides arachnoides*), though another tortoise species, the radiated tortoise (*Astrochelys radiata*), can also be an occasional host (Ehlers et al., 2016). Both tortoise host species are Critically Endangered and endemic to Madagascar. While the biology of *A. chabaudi* is not well studied, it is assumed to have similar life patterns and history as other tortoise-associated *Amblyomma* spp., with the adults feeding exclusively on tortoises and the juvenile stages feeding on both tortoises and other vertebrates (Klompen, 2003). *Amblyomma chabaudi* may also be a health factor for humans and livestock; it is a parasite and also a vector for pathogenic bacteria. Ehlers et al. (2016) found that 100% of sampled *A. chabaudi* ticks were infected with a *Rickettsia* spp., a bacterial genus that is pathogenic to both humans and livestock. There is also concern regarding the spread of *A. chabaudi* outside of Madagascar, as it has been found infesting tortoises in exotic pet trades. In 2002, it was first reported in the United States where it was found to be infesting other reptile hosts in the absence of its preferred host (Burridge and Simmons, 2002).

African Tick-Borne Pathogens

There are many tick-borne pathogens; one pathogen of interest found in *A. chabaudi* is *Rickettsia africae*, the causative agent of African tick bite fever (ATBF). It was originally thought that the disease resulted from infection with *Rickettsia conorii*, the causative agent of Mediterranean spotted fever (MSF) due to the similarities between the diseases. Both Mediterranean spotted fever and African tick bite fever present in humans with a fever, no rash, development of an inoculation eschar, and swollen lymph nodes (Raoult et al., 2001). Pijper et al. (1936) suggested *R. africae* was different from *R. conorii*; Kelly et al. (1992) demonstrated the causative role of *R. africae* in ATBF by isolating it from a patient. African tick bite fever is a milder illness with multiple eschars and swollen lymph nodes, unlike MSF which has only a single eschar, is more severe, and can be fatal (Jensenius et al., 2003). Treatment of ATBF is antibiotic therapy using doxycycline, minocycline, trythromycin, or ciprofloxacin. *Rickettsia africae* is a public health issue, and it is the second most common cause of systemic febrile illness in Africa. While being widespread in Africa, it has also been detected in Niger, Mali, Burundi, Sudan, South Africa, Guadeloupe, and the West Indies. This disease mainly occurs in tourists returning from tick endemic areas (Mediannikov et al., 2010). Dupont et al. (1995) found a 30-56% seroprevalence in sera

collected from patients in 7 different African countries. *Rickettsia africae* is usually transmitted by the tick vectors, *Amblyomma variegatum* and *Hyalomma hebraeum* (Mediannikov et al., 2010) and is maintained in the environment through cattle and other ungulates (Raoul et al., 2001). Though prevalence information of *R. africae* in Madagascar is still lacking, there is a link between habitat degradation and increased infestation of tortoises with *R. africae*-infected ticks (Ehlers et al., 2016). As human encroachment across tortoise habitat increases, more research is needed to prevent any negative consequences to human and livestock health.

Another *Rickettsia* species of interest is *Rickettsia aeschlimannii*, one of the spotted fever group *Rickettsiae* which is normally associated with the reptile tick *Hyalomma marginatum* (Beati et al., 1997). It was first described in 1997 after isolation from a Moroccan *H. marginatum* tick (Beati et al., 1997). Genotypically similar organisms have been detected in other *Hyalomma* ticks from Africa following the initial characterization (Matsumoto et al., 2004). It has been suggested that *R. aeschlimannii* may be another causative agent of MSF (Raoult et al., 2002). The first documented human infection with *R. aeschlimannii* was in 2000; a patient returning from Morocco to France developed a fever, generalized maculopapular skin rash, and an inoculation eschar that became necrotic (Raoul et al., 2002). Infections caused by *R. aeschlimannii* are difficult to distinguish from other rickettsial infections, such as *R. conorii*, based on clinical features alone, especially in areas which have multiple rickettsial pathogens (Matsumoto et al., 2004). Spotted fever group rickettsioses are characterized by headache, high grade fever, cutaneous rash, and eschars, with other nonspecific symptoms during the early stages. Distinguishing characteristics may be absent or unobserved during these early stages (Tosoni et al., 2016). The typical method of differentiation the *Rickettsia* sp. is testing for specific antibodies (Raoult et al., 2002). More severe cases of *R. aeschlimannii* infection are characterized by liver dysfunction and significant hyperaminotransferasemia, a condition in which the liver cells become too permeable and leak enzymes into the bloodstream (Tosoni et al., 2016). *Rickettsia aeschlimannii* may be an important area of public health because of its now broad geographic distribution, having been detected in southern Europe (Tosoni et al., 2016, Fernandez-Soto and Perez-Sanchez, 2003), Germany (Rumer et al., 2011), sub-Saharan Africa (Matsumoto et al., 2004), and China (Wei et al., 2015). The spread of *R. aeschlimannii* in Europe is hypothesized to be associated with migratory birds from Africa (Rumer et al., 2011, Matsumoto et al., 2004). Fernandez-Soto and Perez-Sanchez (2003) showed 6 tick species across 4 genera can carry *R. aeschlimannii*, although *H. marginatum* is considered to be the main vector based on the highest number of infected specimens and highest infection rate.

Borrelia turcica is a reptile-associated *Borrelia.* Reptile-associated *Borreliae* do not phylogenetically align with either the relapsing fever *Borreliae* or Lyme borreliosis groups; they are in their own separate clade (Gofton et a., 2018). However, *B. turcica* does contain relapsing fever-like genes, which may allow it to grow to high densities in blood, similar to the relapsing fever *Borreliae*. Its genome also contains some conserved Lyme borreliosis specific orthologs. These orthologs may provide fitness advantages in the tick midgut environment, with maintenance pathways similar to ones found in *Borrelia burgdoferi sensu stricto* that may play a role in acquisition by, persistence in, and transmission by ticks (Gofton et al., 2018). However, little is known about the biology, ecology, or pathogenicity of *B. turcica*. It has been isolated from tortoises exotic to the US (Takano et al., 2010), with the genus *Testudo* acting as a potential reservoir (Hepner et al., 2020). Hepner et al. (2019) confirmed the potential of tortoises acting as a reservoir by testing the *in vitro* survival of *B. turcica* in turtle, tortoise, human, and bird sera. *Borrelia turcica* had full resistance to tortoise sera and partial resistance to turtle sera, while there was no survival in human or bird sera; this suggests that *B. turcica* is not able to be a human pathogen. Takano et al (2010) experimentally infected tortoises with *B. turcica* and reported that the tortoises showed no symptoms despite developing a systemic infection, being found in skin, whole blood, and muscle tissue. This suggests that tortoises may play a role in maintaining *B. turcica* in the environment. Currently, there is little information on other animals that *B. turcica* may be able to infect.

Malagasy Tortoises

This thesis research focuses on the largest and smallest Malagasy tortoises, the Angonoka tortoise *Astrochelys yniphora* and two spider tortoise subspecies, *Pyxis arachnoides oblonga* and *Pyxis arachnoides arachnoides*. *Astrochelys yniphora* is Critically Endangered (IUCN Red List), found only in Baly Bay National Park in northwest Madagascar (Mandimbihasina et al., 2018). Its large size and "golden" coloration has made it a valuable tortoise in illegal pet trades (Mandimbihasina and Currylow, 2014) where the price can exceed $45,000 USD per tortoise. Exploitation of this tortoise species resulted in a 50% population decline to around 500 breeding pairs in 2014-2015 and the loss of 2 subpopulations. Studies have shown no genetic bottlenecks in the remaining subpopulations, which suggests the tortoise populations may still be healthy. While captive breeding and conservation efforts allowed for the release of over 100 tortoises back into the wild in 2015, breeding is difficult due to the tortoises' delayed age of reproduction of around 17 to 22 years (Mandimbihasina et al., 2019).

Less is known about the biology and ecology of the Critically Endangered *P. a. arachnoides*, spider tortoise, and *P. a. oblonga,* southern spider tortoise (Walker et al., 2008). These are endemic to the southern Madagascar coast, and their range is sympatric with *A. radiata* (Walker et al., 2012), though they prefer coastal spiny forests (Fritz and Havas, 2007). Their range once spanned across 555 km of coastline, but habitat loss has reduced population size to around 30% of historical figures (Walker and Rafeliarisoa, 2012). Their activity is dependent on the rainy seasons; they often go dormant during dry months when there is less lush vegetation. During wet months they are more active and forage, moving to areas of greater vegetation cover (Currylow et al., 2015; Walker et al., 2008). Reproductive behaviors of *P. arachnoides* are still undescribed (Currylow et al., 2015). With both tortoise species being critically endangered, studying ticks collected from them may provide insight into factors that could be detrimental to these tortoises.

METHODS

Tick Collection

From 2012 through 2015, ticks were collected from free-ranging, opportunistically encountered, or radiotracked tortoises during fieldwork on other studies (Currylow, 2016). Ticks were removed from tortoise limbs and scute sutures with a plyer and immediately deposited into a 1.5 mL snap cap microcentrifuge tube filled with 70-91% alcohol. Associated data recorded at the time of collection included tortoise species, tortoise morphometrics, tortoise demographics, tortoise behavior, body location of tick removal, weather, general habitat, date, time, and GPS location. A total of 14 *A. yniphora*, 45 *Pyxis a. arachnoides,* 13 *Pyxis a.s oblonga*, one *A. radiata* were sampled from various locations (Fig. 1 and Fig. 2). All samples were stored at room temperature and transported from Madagascar to the U.S. for analysis. Fieldwork was conducted under the Malagasy Ministry of the Environment and Forests permit numbers 008/13, 009/13, 214/13, 271/13, 112/14, 023/12, 129/14, 005/15, 006/15 and 035/16. All animal-related activities were compliant with University of Southern California IACUC #12046. DNA extracted from *Amblyomma chabaudi* ticks were also received from Germany (generous gift from Julian Ehlers and Professor Jörg Ganzhorn [Universität Hamburg, Hamburg, Germany]).

DNA Extraction

Prior to extraction, adult ticks were cut bilaterally, with one half stored at -80°C and the other half pulverized for extraction. Juvenile ticks were pulverized and extracted whole. Each tick was pulverized using 1 mm glass beads and one 5 mm glass bead in a Mini Beadbeater (Biospec, Inc. Bartlesville, OK, USA) with extractions done using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Extracted DNA was eluted in 100 µL elution buffer.

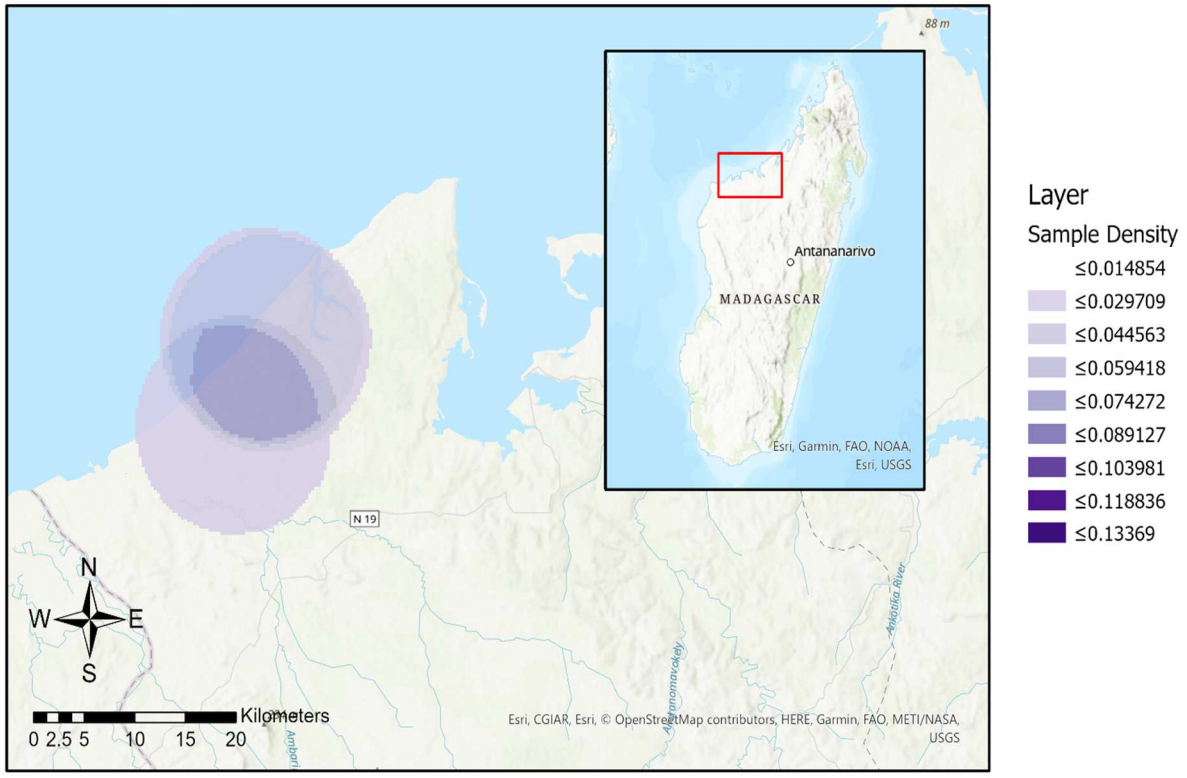

Fig 1. Heat map of the northern Madagascar tortoise sampling locations. Sample density color gradient indicates the overlap of individual tortoise observations in an area.

Molecular Tick Identification

Ticks that could not be morphologically identified were identified using PCR assays that amplified the tick cytochrome oxidase (CO1) and tick 12S rRNA genes (Table 1). Malagasy *Amblyomma chabaudi* tick DNA donated from Germany were also amplified using the aforementioned genes for comparison. Reactions carried out in 25 µL volumes and consisted of 12.5 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 1.25 µL of each primer (10 µM concentration), 5 µL of water, and 5 µL of template DNA. Thermocycler conditions for both CO1 and 12S rRNA were described by Kushimo (2013). PCR products were visualized using gel electrophoresis on a 1.5% agarose gel. Samples that generated a visible band of the correct size were purified using the DNA Wizard Preps Kit according to manufacturer instructions (Promega Corporation, Madison, WI) and sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the aforementioned primers. DNA sequences were assembled in Geneious (Biomatters, San Diego, CA, USA) and compared against known sequences using NCBI BLAST.

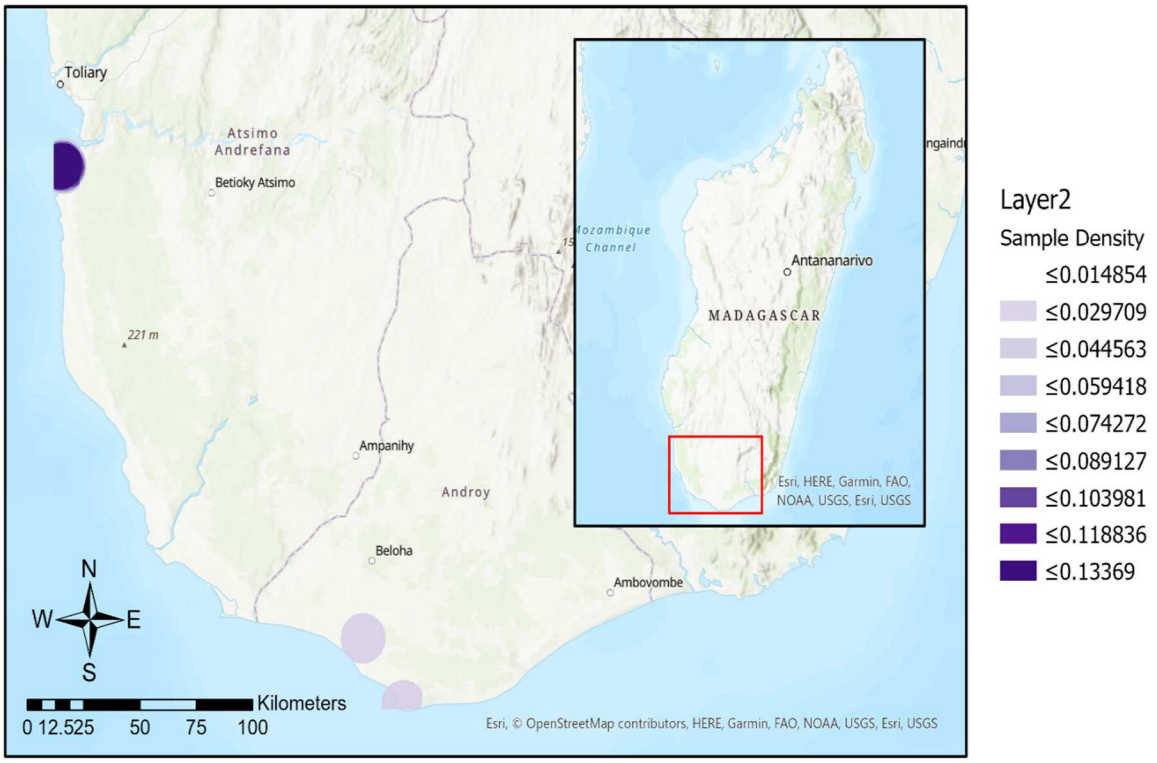

Fig 2. Heat map of the northern Madagascar tortoise sampling locations. Sample density color gradient indicates the overlap of individual tortoise observations in an area.

Table 1. Primers and probes used to determine tick species

Primer/Probe	Primer/Probe Sequence	Reference
Tick 12S rRNA gene		
T1B	5'- AAACTAGGATTAGATACCCT -3'	Kushimo (2013)
T2A	5'- AATGAGAGCGACGGGCGATGT -3'	Kushimo (2013)
Tick CO1 gene		
F1	5'- TACTCTACTAATCATAAAGACATTGG -3'	Kushimo (2013)
R1	5'- CCTCCTCCTGAAGGGTCAAAAAATGA -3'	Kushimo (2013)

Rickettsia spp. Testing and Sequencing

Samples were screened for *Rickettsia* spp. using a real-time PCR assay that amplifies and detects the rickettsial 17 kDA gene (Table 2). Reactions were carried out in 25 µL volumes with 12.5 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 1.25 µL of each primer (10 µM concentration), 1 µL of probe (10 µM concentration), 3.5µL of $MgCl_2$ (25 µM concentration), 0.5 µL of water, and 5 µL of template DNA. Real-time thermocycler conditions were: 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec (Jiang et al., 2012).

Samples positive for *Rickettsia* spp. were amplified using standard end-point PCR for the *Rickettsia* spp. citrate synthase gene (*gltA*) and a nested end-point PCR for the outer membrane protein A gene (*ompA*) (Table 2). Reactions for *gltA* were carried out in 25 µL reactions with 12.5 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 2 µL of each primer (10 µM concentration), 3.5 µL of water, and 5 µL of template DNA. Thermocycler conditions were: 95°C for 3 min, then 40 cycles of 95°C for 15 sec, 48°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 7 min and an indefinite hold at 4°C (Labruna et al., 2004). Outer reactions for *ompA* were carried out in 20 µL volumes with 10 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 1 µL of each primer (10 µM concentration), 6 µL of water, and 2 µL of template DNA. Thermocycler conditions were: 95°C for 5 min, then 40 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, followed by 72°C for 10 min and an indefinite hold at 4°C. Inner reactions for *ompA* were carried out in 20 µL volumes with 10µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 1 µL of each primer (20 µM concentration), 7 µL of water, and 1 µL of the outer reaction product. Thermocycler conditions were the same as the outer reaction (Regnery et al., 1991).

Table 2. Primers and probes used to identify *Rickettsia* spp.

Primer/Probe	Primer/Probe Sequence	Reference
Rickettsia 17kDA		
R17K128F2	5'- GGGCGGTATGAAYAAACAAG -3'	Jiang et al. (2012)
R17K238R	5'- CCTACACCTACTCCVACAAG -3'	Jiang et al. (2012)
R17K202TaqP	5'- 6FAM- CCGAATTGAGAACCAAGTAATGC- 3IABkFQ -3'	Jiang et al. (2012)
Rickettsia gltA		
CS-239	5'- GCTCTTCTCATCCTATGGCTATTAT -3'	Labruna et al. (2004)
CS-1069	5'- CAGGGTCTTCGTGCATTTCTT -3'	Labruna et al. (2004)
*Rickettsia omp*A		
RR190.70 outer	5'- ATGGCGAATATTTCTCCAAAA -3'	Blair et al. (2004)
RR190.701	5'- GTTCCGTTAATGGCAGCATCT -3'	Blair et al. (2004)
RR190.70 inner	5'- ATGGCGAATATTTCTCCAAAA -3'	Regnery et al. (1991)
RR190.622n	5'- AGTGCAGCATTCGCTCCCCCT -3'	Regnery et al. (1991)

Borrelia spp. Testing and Sequencing

Samples were screened for *Borrelia* spp. using a real-time PCR assay that amplifies a fragment of the *Borrelia* 16S rRNA gene as described in Graham et al. (2018). Reactions were carried out in 20 µL reactions with 10 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 1.2 µL of each primer (10 µM concentration), 0.4 µL of probe (10 µM concentration), 2.2 µL of water, and 5 µL of template DNA. Thermocycler conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 45 sec.

Samples positive for *Borrelia* spp. were amplified using nested standard end-point PCR for the *Borrelia* spp. housekeeping genes *uvrA* and *rplB* (Table 3) and the *Borrelia* flagellin gene *flaB* (Table 3). Outer reactions for both housekeeping genes were carried out in 20 µL reactions with 10 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 2 µL of each primer, 5 µL of water, and 1 µL of template DNA. Thermocycler conditions were: a touchdown PCR starting with 95°C for 15 sec followed by 8 cycles of 94°C for 30 sec, 55°C for 30 sec decreasing by 1°C every cycle, and 72°C for 30 sec. An additional 20 cycles were run at 94°C for 30 sec, 48°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 5 min and

an indefinite hold at 4°C (Margos et al., 2008). Inner reactions for the housekeeping genes are identical to the outer reaction using 1 µL of product from the outer reactions. Thermocycler conditions for the inner reactions were: 95°C for 7 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 5 min and an indefinite hold at 4°C (Margos et al., 2008). Outer reactions for *flaB* were carried out in 25 µL reactions with 12.5 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 0.5 µL of each primer (10 µM concentration), 0.75 µL of $MgCl_2$ (50 µM concentration), 5.7 µL of water, and 5 µL of template DNA. Thermocycler conditions were: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec, followed by 72°C for 7 min and an indefinite hold at 4°C (Johnson et al., 1992). Inner reactions were carried out in 25 µL reactions with 12.5 µL of 2X EconoTaq PLUS master mix (Lugien Corp., Middleton, WI), 2 µL of each primer (10 µM concentration), 3.5 µL of water, and 5 µL of outer reaction product diluted 1:10. Thermocycler conditions were: 94°C for 2 min, collowed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, followed by 72°C for 7 min and an indefinite hold at 4°C (Johnson et al., 1992).

Sequence Curation and Phylogeny Analysis

Consensus sequences were generated for each sample from chromatograms with bidirectional coverage of the tick, *Borrelia*, and *Rickettsia* genes of interest. Nucleotide sequences were aligned and curated using Geneious Prime 2020 (https://www.geneious.com). Consensus sequences were compared against known sequences from the NCBI database using BLAST to match identical sequences. Using Geneious Prime 2020, consensus sequences of the *flaB* gene from 6 individual samples were aligned and trimmed to the same length to generate an overall consensus sequence for analysis of other samples. The *flaB* sequences of 4 samples that matched the consensus sequence were concatenated with the *uvrA* and *rplB* sequences for analysis. The *uvrA* and *rplB* sequences of each sample were trimmed to the same length. The *flaB, uvrA,* and *rplB* sequences for *Borrelia miyamotoi, Borrelia afzelii, Borrelia lusitaniae, Borrelia valaisiana, Borrelia bissetti, Borrelia garinii, Borrelia burgdorferi, Borrelia coriaceae, Borrelia parkeri, Borrelia hermsii,* and *Borrelia turcica* were also concatenated to provide comparison to the samples. A phylogenetic tree was then generated using the Tamura-Nei distance model and neighbor-joining build method.

The consensus sequences of the 12S rDNA and CO1 genes of 5 ticks were trimmed to the same length and concatenated to form a phylogenetic tree along with the same regions from 16 previously identified

A. chabaudi generously provided by Julian Ehlers and Professor Jörg Ganzhorn (Universität Hamburg, Hamburg, Germany).

Table 3. Primers and probes used to identify *Borrelia* spp.

Primer/Probe	Primer/Probe Sequence	Reference
Borrelia 16S		
16S-F	5'- AGCYTTTAAAGCTTCGCTTGTAG -3'	Kingry et al. (2018)
16S-R	5'- GCCTCCCGTAGGAGTCTGG -3'	Kingry et al. (2018)
16S-probe	5'-FAM-CGTTCAATACACACATCAAACCACT-3IABkFQ-3'	Kingry et al. (2018)
Borrelia flagellin		
flaB outer 1	5'- AAGTAGAAAAAGTCTTAGTAAGAATGAAGGA -3'	Johnson et al. (1992)
*fla*B outer 2	5'- AATTGCATACTCAGTACTATTCTTTATAGAT -3'	Johnson et al. (1992)
*fla*B inner 1	5'- CACATATTCAGATGCAGACAGAGGTTCTA -3'	Johnson et al. (1992)
*fla*B inner 2	5'- GAAGGTGCTGTAGCAGGTGCTGGCTGT -3'	Johnson et al. (1992)
Borrelia uvrA		
uvrF1408	5'- GAAATTTTAAAGGAAATTAAAAGTAG -3'	Margos et al. (2008)
uvrR2318	5'- CAAGGAACAAAAAACATCTGG -3'	Margos et al. (2008)
uvrF1434	5'- GCTTAAATTTTTAATTGATGTTGG -3'	Margos et al. (2008)
uvrR2111	5'- CCTATTGGTTTTTGATTTATTTG -3'	Margos et al. (2008)
Borrelia rplB		
rplF2	5'- TGGGTATTAAGACTTATAAGC -3'	Margos et al. (2008)
rplR760	5'- GCTGTCCCCAAGGAGACA -3'	Margos et al. (2008)
rplF40	5'- CGCTATAAGACGACTTTATC -3'	Margos et al. (2008)
rplR760	5'- GCTGTCCCCAAGGAGACA -3'	Margos et al. (2008)

RESULTS

Tick infestation of tortoises

Ticks were sampled from a total of 72 tortoises: 14 *A. yniphora,* 44 *P. a. arachnoides*, 13 *P. a. oblonga*, and one *A. radiata*. One hundred ninety-one ticks were removed from *A. yniphora*, 84 from *P. a. arachnoides,* and 21 from *P. a. oblonga*, one from *A. radiata*, and one from the grass. This resulted in a mean of 13.6 ticks per infested *A. yniphora,* 1.9 ticks per infested *P. a. arachnoides*, and 1.6 ticks per infested *P. a. oblonga*, one tick per infested *A. radiata*.

Borrelia and *Rickettsia* Screening

Two hundred thirty-nine ticks were screened for the presence of *Borrelia* spp. and *Rickettsia* spp.; 29 ticks were set aside for archival purposes. One hundred thirty-seven (57.30%) ticks were infected with only *Rickettsia* spp., and 3 (1.39%) were infected with only *Borrelia* spp. Another 43 (18.00%) ticks were co-infected with both *Borrelia* spp. and *Rickettsia* spp. This results in a total of 180 *Rickettsia* spp. infected ticks (75.30%) and a total of 46 *Borrelia* spp. infected ticks (19.30%) (Fig. 3).

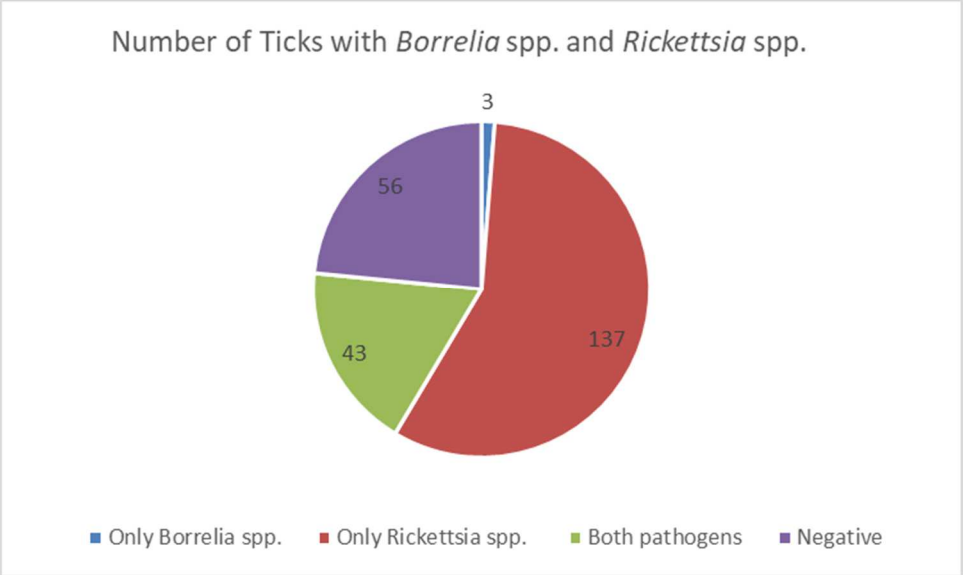

Fig. 3. Number of ticks positive for *Borrelia* spp. and/or *Rickettsia* spp. out of a total of 239 ticks. Results were grouped based on numbers of samples positive for *Rickettsia* spp., *Borrelia* spp., or co-infected with both genera and number of samples negative for bacterial presence.

Identification of *Rickettsia* spp.

To determine the *Rickettsia* spp. present in the ticks, the *gltA* gene was sequenced from 11 *Rickettsia*-positive ticks. BLAST search for similar sequences revealed a 99.7-100% identity, over 686 nucleotides, to *R. africae* isolate HuAvRcgltA (GenBank accession MT905433) for 4 samples. Seven samples had a 99.7-99.8% identity, over 810 nucleotides, to *R. aeschlimannii* isolate Raeschlimannii_Tick15 (GenBank accession MH267736).

Borrelia Genetic Diversity

Ticks positive for *Borrelia* spp. had the *flaB* gene amplified and sequenced; 45 ticks had a 95-98% identity, over 427 nucleotides, to *Borrelia turcica flaB*. One sample was unable to have its *flaB* gene amplified. The *uvrA* housekeeping gene was amplified for 20 ticks; all had 94-95% identity, over 844 nucleotides, to the *B. turcica* IST7 strain. Nine samples were sequenced for the *rplB* housekeeping gene and had a 94-95.3% identity, over 508 nucleotides, to the *B. turcica* IST7 strain. A phylogenetic analysis for 5 samples that amplified all 3 genes, based on a concatenation of the genes, showed one tick (TC0164813MC301) associated with the relapsing fever group, one (TC02371415MC402) was in between *Borrelia miyamotoi* and the Lyme borreliosis group, and 3 (TC0156918MC401, TC0327515MC401, TC03172214MX401) had a close relationship with *B. turcica* (Fig. 4). Based on the concatenation of only 2 genes, the *flaB* and *uvrA,* 6 samples showed a close relationship to *B. turcica* (Fig. 5). Individual phylogenetic analysis of all 3 genes was done on a small number of samples. Analysis showed that 6 samples sequenced for *flaB* showed a close relationship with *B. turcica* (Fig. 6), and 6 samples sequenced for *uvrA* showed a close relationship with *B. turcica* (Fig. 7). Four samples were used to create individual *rplB* phylogenetic trees. Three samples (TC02371415MC402, TC0327515MC401, TC03172214MX401) had a close relationship with *B. turcica* and one sample (TC0164813MC301) clustered with the relapsing fever group (Fig. 8).

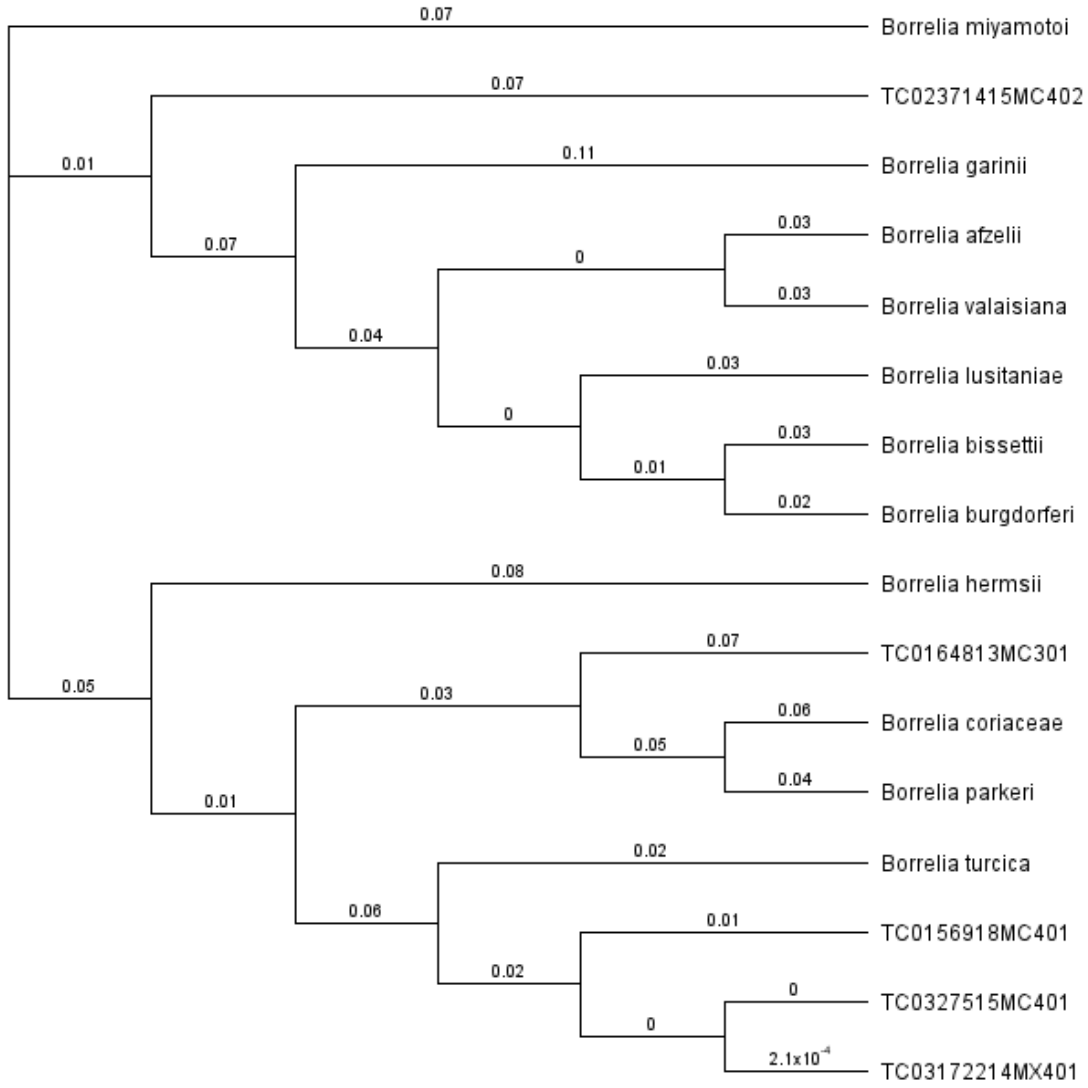

Fig. 4. Phylogenetic analysis based on the concatenation of *flaB*, *uvrA,* and *rplB* of the genus *Borrelia*. This analysis shows one tick (TC0164813MC301) associated with the relapsing fever group, one (TC02371415MC402) in between *Borrelia miyamotoi* and the Lyme borreliosis group, and three (TC0156918MC401, TC0327515MC401, TC03172214MX401) with a close relationship to *B. turcica*.

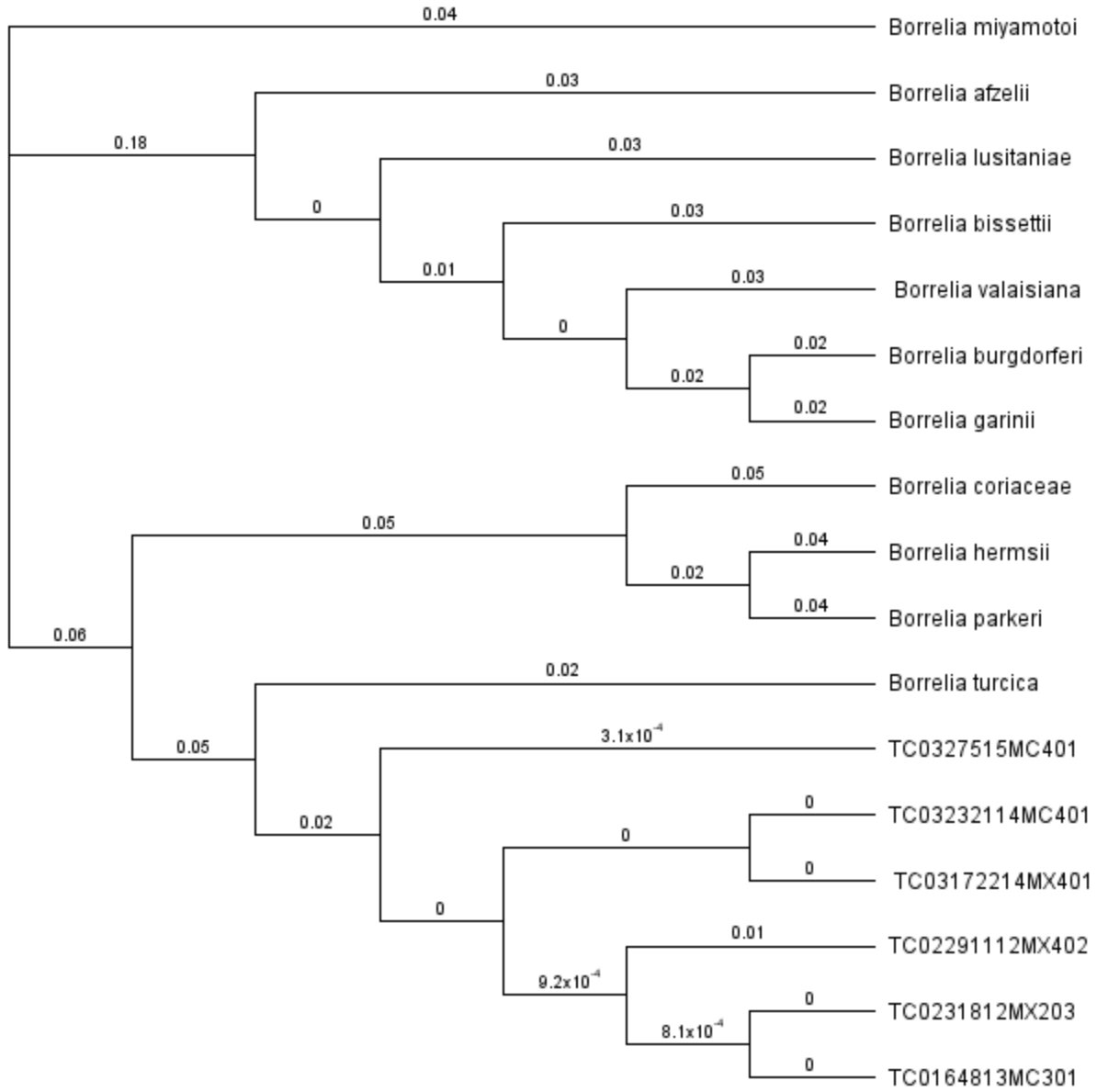

Fig. 5. Phylogenetic analysis based on the concatenation of *flaB* and *uvrA* of the genus *Borrelia* shows a close relationship to *B. turcica*.

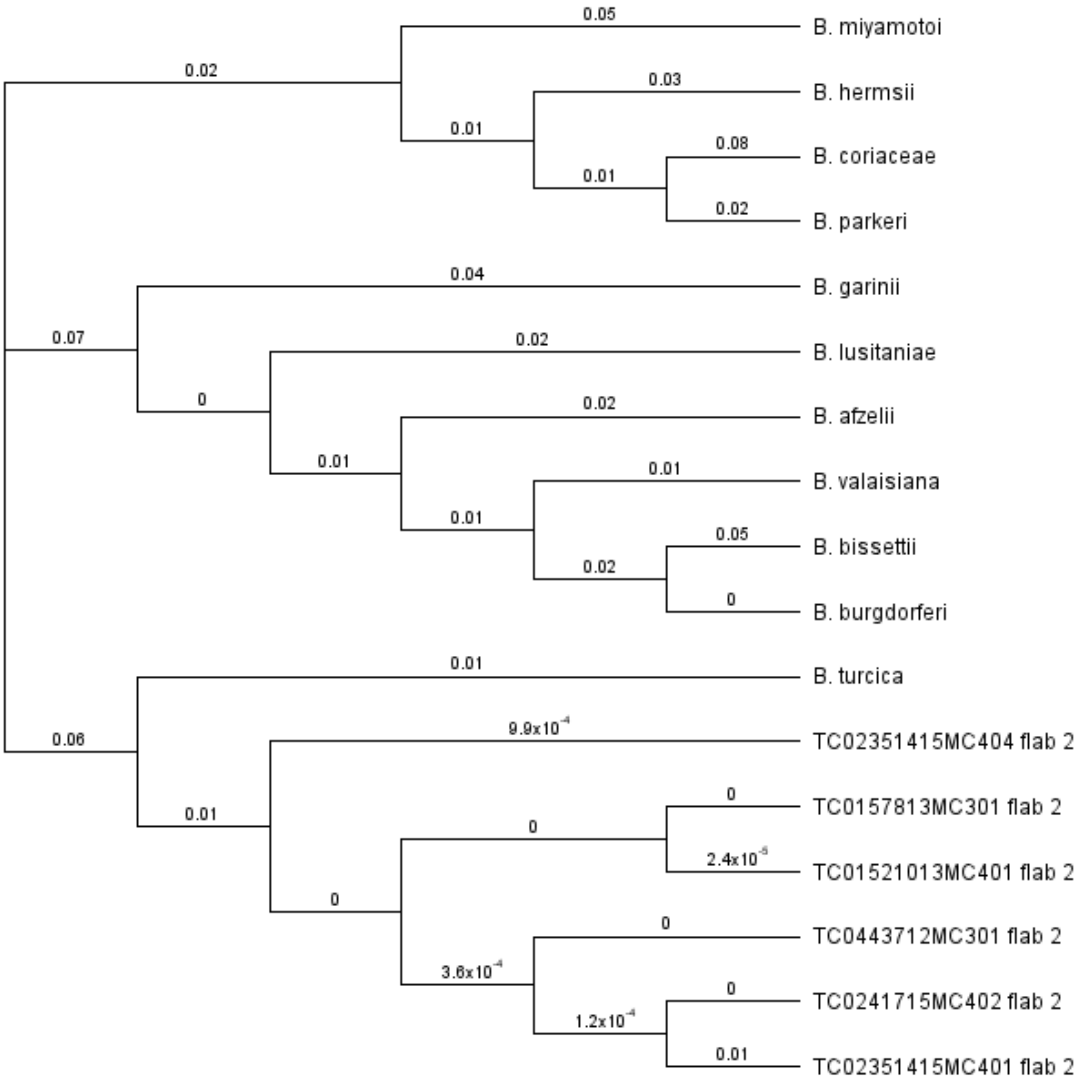

Fig. 6. Phylogenetic analysis based on the amplification of *flaB* of the genus *Borrelia* shows a close relationship to *B. turcica*.

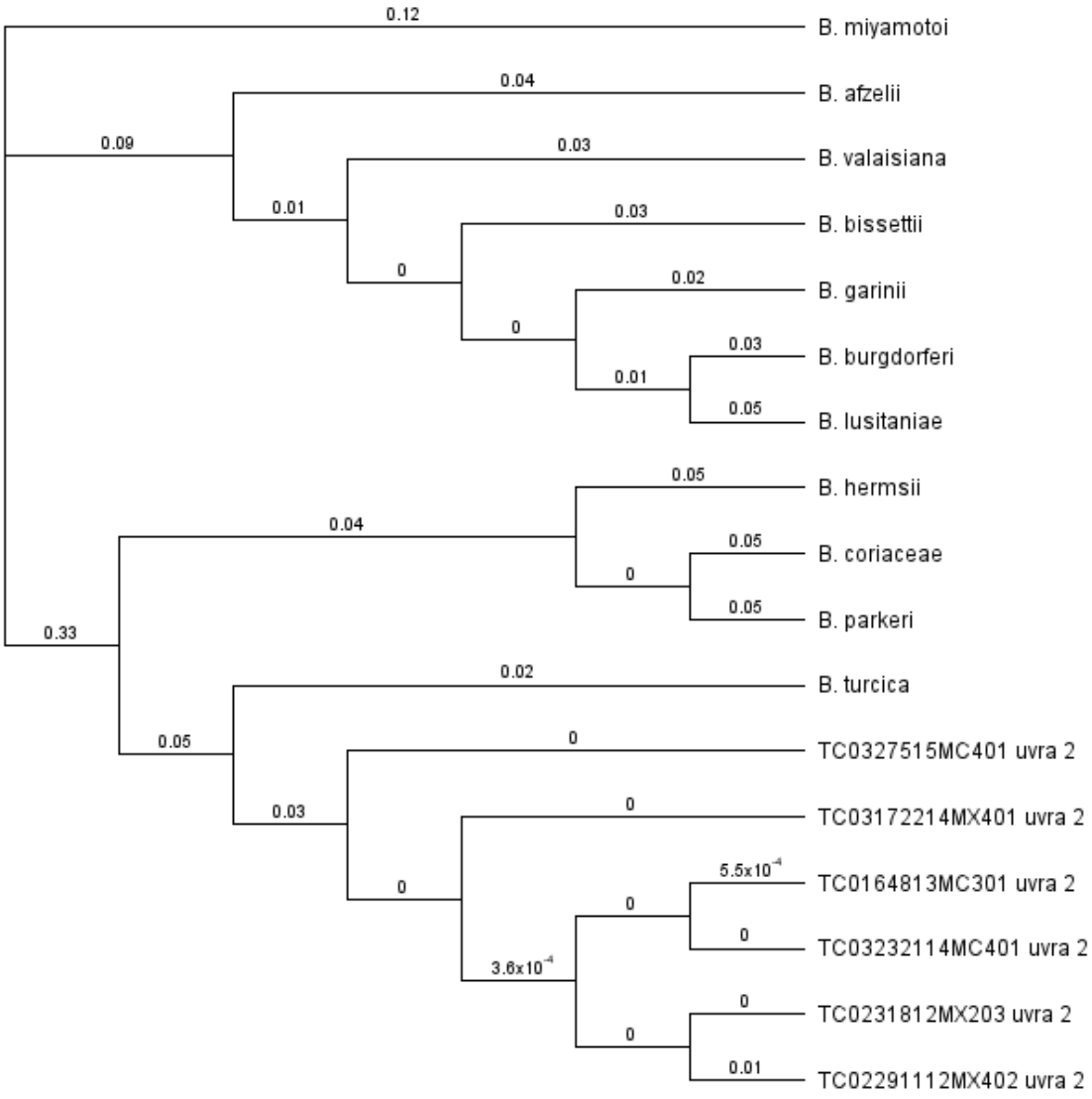

Fig. 7. Phylogenetic analysis based on the amplification of *uvrA* of the genus *Borrelia* shows a close relationship to *B. turcica*.

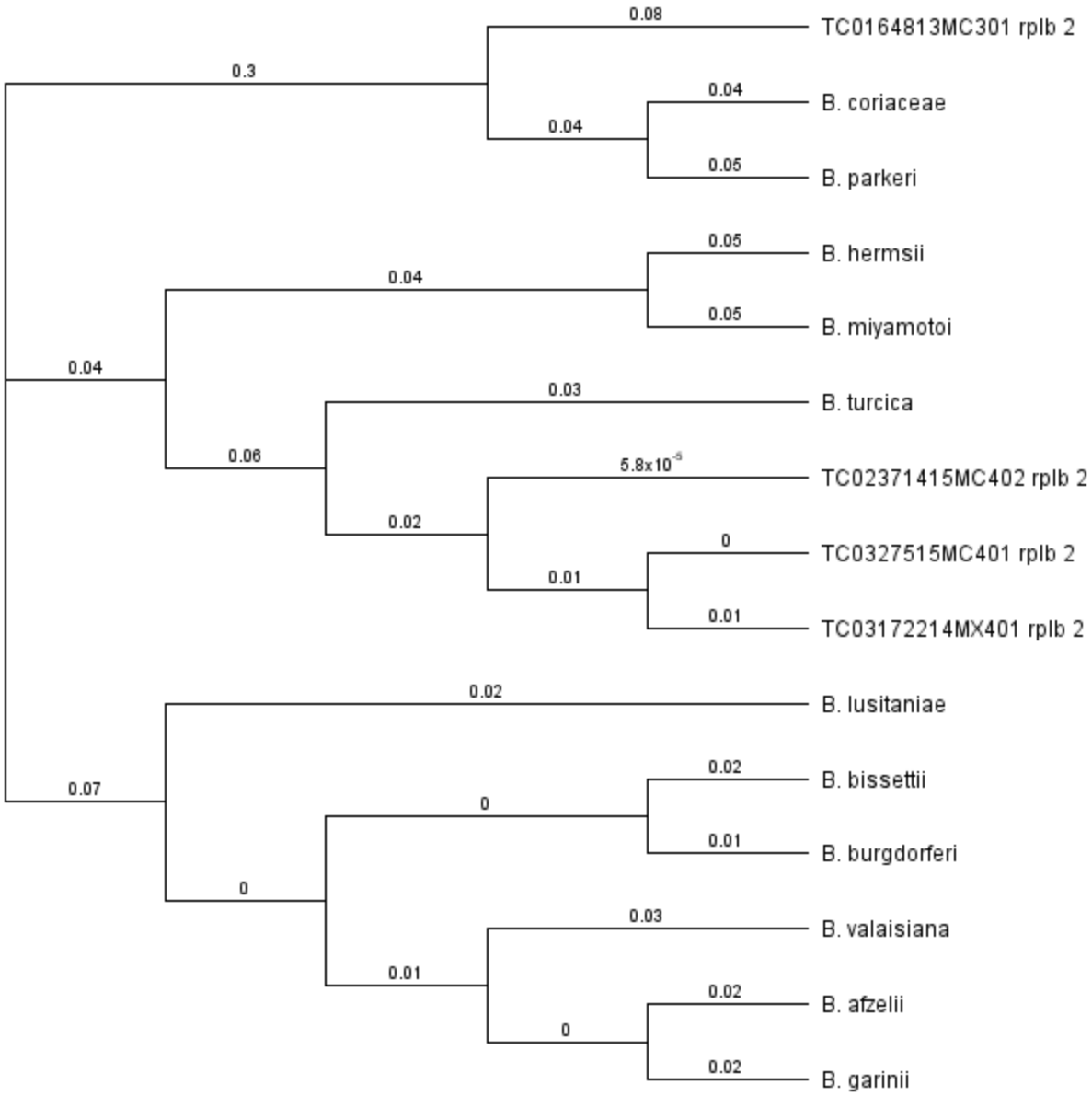

Fig. 8. Phylogenetic analysis based on the amplification of *rplB* of the genus *Borrelia*. This analysis shows one tick (TC0164813MC301) clustered with the relapsing fever group and the three others (TC02371415MC402, TC0327515MC401, TC03172214MX401) with a close relationship to *B. turcica*.

Tick Identification

A total of 83 ticks were morphologically identified as *A. chabaudi*, 5 as *A. geochelone,* 2 as *A. variegatum*, and 208 as unknown. Sixty-two of the morphologically identified *A. chabaudi* ticks were collected from 33 *P. a. arachnoides* tortoises, 15 were collected from 10 *P. a. oblonga* tortoises, 4 were collected from one *A. yniphora* tortoises, and one collected from one *A. radiata*. Three of the morphologically identified *A. geochelone* ticks were collected from 2 different *A. yniphora* tortoises and the other 2 were collected from one *P. a. arachnoides* tortoise. The two *A. variegatum* ticks each came from a different *P. a. arachnoides* tortoise.

Twenty-eight samples, including both morphologically identified ticks and unknown ticks, were sequenced for the 12S rRNA gene. Of the 28 samples, 20 were sequenced in both directions while 7 were sequenced in either the reverse or forward direction. A BLAST search for similar sequences revealed a 92-94.1% match to *Amblyomma* sp. B MDL (GenBank accession KC817417) for all samples that were sequenced. The 16 samples provided by the German group, that were previously identified as *A. chabaudi,* also had approximately a 92% match to *Amblyomma* sp. B MDL. An alignment of the 16 German-provided samples with 12 of this study's samples for 12S rRNA showed that 5 of this study's samples differed from the German-provided samples by only 2 nucleotides over 355 nucleotides. The other 7 samples had only a 60.8% match to the other 5 samples (Table 4), which was confirmed via phylogenetic analysis (Fig. 9).

Table 4. Degree of similarity between the 12S rRNA sequences of 12 samples of 12 tortoises

	TC0178 1613M X307	TC0184 1215M X401	TC0171 1215M B301	TC0165 1215M B406	TC0165 1215M B304	TC0463 1213M C402	TC0239 1415M C401	TC0162 1215M A202	TC0160 1713M X406	TC0160 1713M X405	TC0160 1713M A201	TC015013 15MX403
TC01501315	60.77	60.77	60.77	60.77	60.77	99.39	99.39	100	100	100	100	
TC01601713	60.77	60.77	60.77	60.77	60.77	99.39	99.39	100	100	100		100
TC01601713	60.77	60.77	60.77	60.77	60.77	99.39	99.39	100	100		100	100
TC01601713	60.77	60.77	60.77	60.77	60.77	99.39	99.39	100		100	100	100
TC01621215	60.77	60.77	60.77	60.77	60.77	99.39	99.39		100	100	100	100
TC02391415	60.77	60.77	60.77	60.77	60.77	100		99.39	99.39	99.39	99.39	99.39
TC04631213	60.77	60.77	60.77	60.77	60.77		100	99.39	99.39	99.39	99.39	99.39
TC01651215	100	100	100	100		60.77	60.77	60.77	60.77	60.77	60.77	60.77
TC01651215	100	100	100		100	60.77	60.77	60.77	60.77	60.77	60.77	60.77
TC01711215	100	100		100	100	60.77	60.77	60.77	60.77	60.77	60.77	60.77
TC01841215	100		100	100	100	60.77	60.77	60.77	60.77	60.77	60.77	60.77
TC01781613		100	100	100	100	60.77	60.77	60.77	60.77	60.77	60.77	60.77

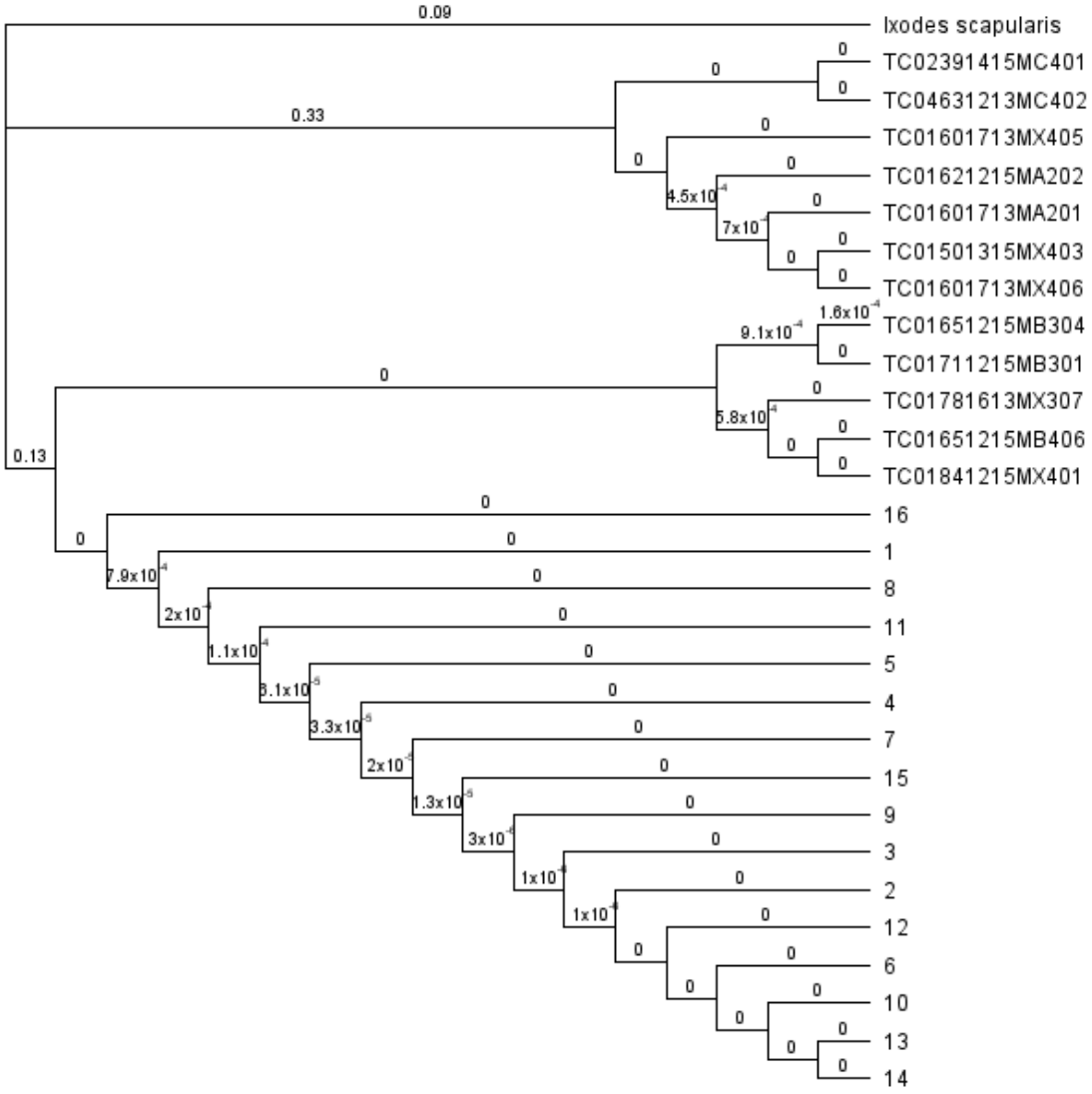

Fig. 9. Phylogenetic analysis based on tick 12S rRNA. The phylogenetic tree was generated using the Tamura-Nei distance model and neighbor-joining build method and confirms that 5 of this study's samples differed from the German-provided samples by only 2 nucleotides over 355 nucleotides while the other 7 samples had only a 60.8% match to those 5 samples. The bar indicates scale of sequence divergence. *Ixodes scapularis* (HG918113.1) was used as the outgroup.

Only 6 samples were sequenced using the CO1 gene; a BLAST search revealed an 85.3-86.4% match to *Amblyomma marmoreum* clone 1M_14677_125 (GenBank accession KY457516). An alignment of the CO1 sequences showed that this study's samples differed from each other by only 2 nucleotides, over 552 nucleotides, and the consensus sequence differed from the German-provided samples by only 2 nucleotides, over 552 nucleotides.

Tick-Pathogen-Tortoise Relationship

The 46 *Borrelia*-positive ticks were collected from 37 tortoises. Seven of these ticks were collected from 7 different *A. yniphora* tortoises; one tick was morphologically identified as *A. chabaudi* while the other 6 were unknowns. Thirty-three ticks were collected from 24 different *P. a. arachnoides* tortoises; twenty-four of these ticks were morphologically identified as *A. chabaudi* while the other 9 were unknowns. Six ticks were collected from 6 different *P. a. oblonga* tortoises; four were morphologically identified as *A. chabaudi* and the other 2 were unknowns (Appendix).

The 180 *Rickettsia*-positive ticks were collected from 63 tortoises. One hundred twenty-one of these ticks were collected from 26 different *A. yniphora* tortoises; one tick was morphologically identified as *A. chabaudi*, one morphologically identified as *A. geochelone,* and the other 119 were unknown. Forty-six ticks were collected from 27 different *P. a. arachnoides* tortoises; thirty-one ticks were morphologically identified as *A. chabaudi* while the other 15 were unknowns. Eleven ticks were collected from 8 different *P. a. oblonga* tortoises; nine ticks were morphologically identified as *A. chabaudi* while the other 2 were unknowns. One morphologically identified *A. chabaudi* tick was collected from one *A. radiata* tortoise, and one unknown tick was collected from grass (Appendix).

Forty-three of the infected ticks were co-infected with both *Borrelia* spp. and *Rickettsia* spp.; these ticks were collected from 31 tortoises. Six of these ticks were collected from 5 different *A. yniphora* tortoises; one tick was morphologically identified as *A. chabaudi* and the other 5 were unknown. Thirty-three ticks were collected from 22 different *P. a. arachnoides* tortoises; twenty-four ticks were morphologically identified as *A. chabaudi* while the other 8 were unknowns. Four ticks were collected from 4 different *P. a. oblonga* tortoises; three ticks were morphologically identified as *A. chabaudi* while the remaining tick was unknown (Appendix).

DISCUSSION

Madagascar is unique in its biological diversity and level of endemism due to its isolated island habitat. New species continue to be discovered, but human encroachment has the potential to disrupt the natural ecosystem balance by bringing new fauna and flora to this island. Ticks, such as *Amblyomma variegatum,* native to the African mainland, have been reported in Madagascar (Barré et al., 1995). Ticks are an important vector for many zoonotic pathogens, such as *Rickettsia africae*, the causative agent of African tick-bite fever (Mediannikov et al., 2010). While tick-transmitted pathogens pose a health threat to humans, they may also pose a threat to the Malagasy tortoises, *P. arachnoides* and *A. yniphora*. These tortoises are Critically Endangered, making them an important focus for conservation. Studying the relationship of the tortoises with ectoparasites can help determine any infection risk.

This study focused on the relationship between ticks, their pathogens, and tortoises found in Madgascar. In the sampled tortoises, tick infestation rates were higher in *A. yniphora* (13.6/tortoise) than the other tortoise hosts (1.20-1.61/tortoise). However, the body condition of the tortoises was not available, so conclusions on the effect of high tick infestations on host health could not be drawn.

Morphological identification based on the scutum ornamentation of the collected ticks determined that 59 were the tortoise tick *Amblyomma chabaudi*, a common ectoparasite of the endemic spider tortoise *P. arachnoides* (Ehlers et al., 2016). However, many samples were marked unknown due to deterioration of the scutum ornamentation, most likely caused by prolonged exposure to the alcohol the samples were stored in. Molecular analysis of 12 ticks and DNA from 16 previously identified *A. chabaudi* ticks showed that there was no good match for comparison in the NCBI GenBank database. According to the phylogenetic tree generated by the 12S rRNA gene sequences of these 28 samples, there was a clade of 7 samples that clustered in a different branch than the German-provided samples, suggesting that they may not be *A. chabaudi*, despite 2 of them being previously as *A. chabaudi* following the description of Uilenberg (1965). Other ticks in that clade were morphologically identified as *Amblyomma geochelone* by comparing them to inked drawings of Durden et al. (2002). In the phylogenetic tree generated by the 12S rRNA gene sequences, 3 of the morphologically identified *A. geochelone* ticks clustered with the previously identified *A. chabaudi* ticks from Germany. This result agrees with those of Kushimo (2013),

who had found that *A. geochelone* and *A. chabaudi* grouped together in a clade with a bootstrap value of 100, indicating that these two species may belong to a single taxon. However, due to a lack of sequences for the tick 12S and CO1 genes for these species in GenBank as well as the lack of sufficient morphological descriptions of *A. chabaudi*, the identity of many samples is still unknown.

Amblyomma chabaudi have been reported to feed exclusively on the spider tortoise (Klompen, 2003, Ehlers et al., 2016). A majority of the morphologically identified *A. chabaudi* ticks from this study were collected from both subspecies of *P. arachnoides* (91.5%), however this study found that *A. chabaudi,* similar to the German *A. chabaudi* clade, can also feed on *A. yniphora*, indicating that knowledge on the life history of *A. chabaudi* remains incomplete. One *A. chabaudi* was also collected from one *A. radiata* tortoise, verifying that *A. chabaudi* can feed on *A. radiata* in the wild; however, the number is too low to make an estimate of *A. chabaudi* infestation of *A. radiata*. It can be hypothesized that migratory birds may play a role in the spread of these ticks, as *P. arachnoides* and *A. radiata* are endemic to the southern region of Madagascar, while *A. yniphora* is endemic to a small section of northern Madagascar. Anthropogenic effects, such as agricultural encroachment onto tortoise habitats and human travel, may also play a role in the movement of ticks between the 3 tortoise species. Other tortoise-associated juvenile ticks are not as host specific as adults, feeding on both small and large mammals (Pastiu et al., 2012); small mammals and birds may be potential hosts to these juvenile ticks if their life history is similar to that of other tortoise-associated ticks (Klompen, 2003).

The ticks' potential as a vector for pathogens was investigated by using two pathogens commonly found in ticks, *Borrelia* and *Rickettsia.* In contrast to a study done by Ehlers et al. (2016) who reported only *Rickettsiae*, this study found both *Borreliae* and *Rickettsiae*. A high prevalence of *Rickettsia* spp. (75.31%) was found in the ticks tested compared to the prevalence of *Borrelia* spp. (19.25%). Ehlers et al. only screened for relapsing fever *Borreliae*, which may be why no *Borrelia* spp. were detected. The *Rickettsia* spp. found in this study were *Rickettsia africae* and *Rickettsia aeschlimannii*; both species belong to the pathogenic spotted fever group of *Rickettsia* (Kelly et al., 1997, Beati et al., 1997). The high prevalence of *Rickettsia* spp. was expected, as Ehlers et al. (2016) also found a high prevalence of *Rickettsia* spp. (100%).

A phylogenetic tree generated by the concatenation of the genes *flaB, uvrA,* and *rplB* surprisingly showed 2 of the *Borrelia*-positive ticks used clustered with *B. turcica* while the other 2 clustered with

the relapsing fever group, despite having a similar percent match to *B. turcica* (94-97%) for all 3 genes. The phylogenetic tree generated by the concatenation of just the *flaB* and *uvrA* genes showed that all samples used clustered with *B. turcica*, as expected. Differences in the phylogenetic trees may be due to the smaller number of *rplB* sequences available, as many samples failed to initially amplify *rplB*. Individual phylogenetic trees generated for each gene revealed a more distinct difference between *B. turcica* and the samples, the relapsing fever group, and the Lyme borreliosis group. The tree generated by *flaB* sequences (Fig. 6) showed the 6 samples that were included were closely related to *B. turcica*, although they formed their own clade, which was expected due to the 95-98% similarity to *B. turcica flaB*. The tree generated by the *uvrA* (Fig. 7) sequences of 6 other samples showed similar results, with the samples forming their own clade though still closely related to *B. turcica.* The tree generated by *rplB* sequences (Fig. 8), however, did not distinguish between the relapsing fever group and *B. turcica* as well as the other trees. Two of the relapsing fever *rplB* sequences, *Borrelia hermsii* and *Borrelia miyamotoi*, formed a different clade from the other two relapsing fever *Borreliae*, *Borrelia coriaceae* and *Borrelia parkeri*. According to the *rplB* phylogenetic tree (Fig. 8), both *B. hermsii* and *B. miyamotoi* were more closely related to *B. turcica*, which was on its own branch. That tree showed similar results to the tree based on the concatenation of all 3 genes (Fig. 4); three samples clustered with *B. turcica* while one clustered with relapsing fever *Borreliae*. Differences between the *rplB* and the *uvrA* and *flaB* trees may be due to the small sample size.

The relationship between ticks, their pathogens and tortoises is poorly studied. Though *A. chabaudi* was expected to be commonly found on the spider tortoises *P. a. arachnoides* and *P. a. oblonga* (91.5%), the majority of the unknown ticks were collected from *A. yniphora* (71.3%). Human encroachment and proximity to tortoise habitat may factor into the increase of ticks infesting *A. yniphora.* Ehlers et al. (2016) found that tick prevalence on *P. arachnoides* positively correlated with tortoises in disturbed habitats. Baly Bay National Park, the natural range of *A. yniphora*, has been increasingly impacted by anthropogenic bush fires set for cattle grazing (Kiester et al., 2013). This current study finds no obvious relationship between tortoise species and the number of infected ticks collected from the tortoises. Ticks infected with *Rickettsia* spp. and/or *Borrelia* spp. were found on 3 of the 4 tortoise species/subspecies, with 87.5% of *A. yniphora*, 87.1% of *P. a. arachnoides*, and 76.9% of *P. a. oblonga* carrying infected ticks. Conclusions on the relationship between tick species and presence of *Rickettsia* spp. and *Borrelia* spp. could not be made due to the high number of unidentified ticks.

Although tortoises are not seen as a significant reservoir of human pathogens, the ticks they carry can carry a variety of human and livestock pathogens (Ehlers et al., 2016). Many ticks collected from tortoises in this study were infected with pathogenic *Rickettsia* spp. While there is little knowledge on the effects of rickettsial pathogens on tortoises, these tortoises may carry infected ticks into areas where contact with other vertebrates occurs; this may be important as juvenile tortoise ticks are not as host specific as adults (Pastiu et al., 2012). This presents both a public health and veterinary health issue since infected ticks may transmit *R. africae* and *R. aeschlimannii* to either livestock and/or humans.

There was a much lower prevalence of *Borrelia* spp. than *Rickettsia* spp. in tortoises tested in this study; but again there is little knowledge on the effects of *Borrelia* spp. on tortoises. Though a reptile-associated pathogen, there is little definitive knowledge on the ecology and pathogenicity of *B. turcica* in tortoises. Phylogenetic analysis in this study indicates that *B. turcica* may be similar to relapsing fever *Borreliae,* as they seem to have diverged from a common ancestor. Kalmar et al. (2015) isolated *B. turcica* from *Hyalomma aegyptium* ticks collected from the tortoise *Testudo graeca*. Blood samples taken from *T. graeca* showed an absence of *B. turcica* in tortoise blood, suggesting that the natural host of *B. turcica* may be something else, such as small mammals, rather than the tortoises. However, this conflicts with the results from Takano et al. (2010) who showed that *B. turcica* was able to be isolated from *T. graeca* tortoises. Takano et al. (2010) also showed the absence of *B. turcica* in mice and asymptomatic systemic infection in *Geochelone sulcate* tortoises, further conflicting with the results of Kalmer et al. (2015). In this current study, observations of tortoise health and blood samples were not included, so conclusions could not be drawn on bacterial levels and the manifestation of any symptomatic infection in *Rickettsia* and/or *Borrelia*-positive tortoises.

The results of this study and other studies indicate that tick-pathogen-tortoise interactions require more investigation. In this study, no significant relationship was observed between the rate of tick infestation and tortoise species. However, disturbance of natural tortoise habitats from human agricultural activities have impacted the rate of contact between native species and domestic animals (Ehlers et al., 2016). The habitats of *P. arachnoides* and *A. yniphora* are especially threatened by cattle grazing (Ehlers et al., 2016; Kiester et al., 2013), and increased contact with humans may be linked with increased tick infestation of both tortoises and livestock. The ticks in this study have the potential to carry both *Rickettsia* spp. and *Borrelia* spp., which presents as risk to humans and livestock, as these are known to be pathogenic. However, little is known about the effects of these pathogens on tortoises and more

investigation is needed, especially with regards *B. turcica* since its pathogenicity in different tortoises and therefore zoonotic potential is unknown. Further investigation on the pathogenicity of both *B. turcica* and rickettsial pathogens would especially be useful in the conservation of Malagasy tortoises.

CONCLUSION

Despite being a species rich and diverse taxon important to various ecosystems, reptiles are understudied (Czech et al., 1998). Many species are at risk for extinction in part to disease-induced declines resulting from emerging diseases (Bower et al., 2019). Ticks, common ectoparasites and well-known vectors of various pathogens, are often focused on in public health and veterinary implications that can arise from the pathogens they carry (Sonenshine et al., 2002). However, there is little information on the relationship between reptiles and ticks and their parasites (Burridge, 2001). Three species of Malagasy tortoises, *A. yniphora, A. radiata, P. arachnoides,* are Critically Endangered (IUCN Red List) and at risk from habitat loss (Walker and Rafeliarisoa, 2012) and illegal pet trade (Mandimbihasina and Currylow, 2014). This study focused on ticks collected from observed tortoises and the pathogens carried by these ticks.

Ticks were morphologically identified and then checked molecularly to confirm identification or identify any ticks that could not be morphologically identified. Molecular identification was done by end-point PCR that amplified two tick genes, CO1 (cytochrome oxidase) and 12S rRNA. Tick gene sequences were analyzed alongside morphologically confirmed *A. chabaudi* ticks. Ticks were also screened for the presence of *Rickettsia* spp. and *Borrelia* spp. through 2 real-time PCR assays, amplifying the rickettsial 17 kDA and *Borrelia* 16S rRNA genes. Ticks positive for either pathogen were analyzed by end-point PCR sequencing to determine the species, using the rickettsial *gltA* and *Borrelia flaB, uvrA,* and *rplB* genes.

Two hundred ninety-eight ticks were collected from 72 different tortoise hosts. Eighty-three ticks were morphologically identified as *A. chabaudi*, 5 as *A. geochelone*, 2 as *A. variegatum*, and 208 unknowns. A group of morphologically identified ticks and unknowns were sequenced alongside the German-provided samples, although no definite identification was made through BLAST results. A phylogenetic tree based on the 12S rRNA sequences of 12 samples plus the 16 German-provided samples showed that 3 *A. geochelone* ticks clustered with the German samples while 2 *A. chabaudi* ticks formed a different clade with 5 other unknowns. Two hundred thirty-nine ticks were screened for the presence of *Rickettsia* spp. and *Borrelia* spp., with 183 ticks positive for either one or both pathogens. *Rickettsia aeschlimannii* and *R. africae* were sequenced from the *Rickettsia*-positive ticks, and a *Borrelia* species related to *B. turcica* was sequenced from the *Borrelia*-positive ticks. Phylogenetic analysis of the *flaB*

and *uvrA* sequences showed that the samples clustered with *B. turcica*; *rplB* sequences showed that 3 samples clustered with *B. turcica* and one sample clustered with the relapsing fever *Borreliae*.

Amblyomma chabaudi is reported to feed exclusively on *P. arachnoides* (Klompen, 2003). In this study, however, while a majority of the morphologically identified *A. chabaudi* were collected from *P. arachnoides,* there were a number of *A. chabaudi* that were collected from *A. yniphora* and one from *A. radiata*. Information on the life history of *A. chabaudi* is lacking, but the loss of tortoise habitat for agriculture and human travel may play a role in the spread of ticks between these 3 tortoise species. This creates a potential health issue for both humans and livestock, as human and agricultural encroachment into tortoise habitat increases the rate of contact between native animals and domestic animals. The ticks in this study have the potential to carry both *Rickettsia* spp. and *Borrelia* spp., which can present an issue to human and veterinary health, as infected ticks can transmit these pathogens into humans and livestock. However, the information on the relationship between tortoises and tick pathogens is lacking, as little is known about the effects of rickettsial infections on tortoises and *B. turcica* infections vary between tortoise species. Further investigation on tick-pathogen-tortoise interactions are needed to better understand the relationship between ticks and tortoises and how that knowledge can be used in the conservation of Malagasy tortoises.

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APPENDIX

Ticks tested for the presence of *Rickettsia* spp. and *Borrelia* spp. and the tortoise host they were collected from

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC02351415MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0241715MC403	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0230712MC403	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC0232715MC301	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0233913MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0230712MC302	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC0148515MC401	*A. radiata*	N	Y	*A. chabaudi*
TC0241715MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC01521013MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0157813MC301	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0443712MC301	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC02351415MC404	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC04471215MC301	*P. arachnoides oblonga*	Y	Y	*A. chabaudi*
TC04491215MC401	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC0459913MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0459913MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0236715MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0155913MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0154813MC301	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC01532014MC401	*A. yniphora*	Y	Y	*A. chabaudi*
TC02341012MC401	*P. arachnoides oblonga*	Y	Y	*A. chabaudi*
TC0156918MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0444712MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC04461613MX301	N/A- in grass	N	Y	unknown
TC01421713MX401	*A. yniphora*	N	Y	unknown
TC01601713MX402	*A. yniphora*	N	Y	unknown
TC01601713MA201	*A. yniphora*	N	N	unknown
TC0241715MX301	*P. arachnoides arachnoides*	Y	Y	unknown
TC01621215MA202	*A. yniphora*	N	N	unknown
TC01601713MX406	*A. yniphora*	N	N	unknown
TC0216712MX301	*P. arachnoides arachnoides*	Y	Y	unknown
TC01515013MX401	*A. yniphora*	N	Y	unknown
TC01501315MX403	*A. yniphora*	N	N	unknown
TC01601713MX407	*A. yniphora*	N	Y	unknown
TC01601713MX405	*A. yniphora*	N	N	unknown
TC01611215MX402	*A. yniphora*	N	Y	unknown

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC01501315MX402	*A. yniphora*	Y	Y	unknown
TC01581215MX301	*A. yniphora*	N	Y	unknown
TC01581215MA202	*A. yniphora*	N	Y	unknown
TC01601713MX404	*A. yniphora*	N	N	unknown
TC0218712MX301	*P. arachnoides arachnoides*	N	Y	unknown
TC0215712MX401	*P. arachnoides arachnoides*	N	Y	unknown
TC01611215MX301	*A. yniphora*	N	Y	unknown
TC01621215MX401	*A. yniphora*	N	Y	unknown
TC01601713MX403	*A. yniphora*	N	Y	unknown
TC01454913MX301	*A. yniphora*	N	Y	unknown
TC0216712MX401	*P. arachnoides arachnoides*	N	Y	unknown
TC03172214MX401	*P. arachnoides oblonga*	Y	N	unknown
TC02291112MX301	*P. arachnoides arachnoides*	Y	Y	unknown
TC01501315MX301	*A. yniphora*	N	Y	unknown
TC01651215MX301	*A. yniphora*	N	Y	unknown
TC01651215MX402	*A. yniphora*	N	Y	unknown
TC01651215MB304	*A. yniphora*	N	N	*A. geochelone*
TC01651215MX407	*A. yniphora*	N	Y	unknown
TC01651215MX408	*A. yniphora*	N	N	unknown
TC01651215MX409	*A. yniphora*	N	N	unknown
TC1651215MX408	*A. yniphora*	N	N	unknown
TC01651215MB406	*A. yniphora*	N	Y	*A. geochelone*
TC01651215MX405	*A. yniphora*	N	Y	unknown
TC01651215MX403	*A. yniphora*	N	Y	unknown
TC04631213MC401	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC0164813MC301	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC01651215MX410	*A. yniphora*	N	Y	unknown
TC03232114MC401	*P. arachnoides oblonga*	Y	Y	*A. chabaudi*
TC0225812MX202	*P. arachnoides arachnoides*	Y	Y	unknown
TC0231812MX204	*P. arachnoides arachnoides*	N	Y	unknown
TC03264413MX201	*P. arachnoides oblonga*	N	N	unknown
TC03264413MX203	*P. arachnoides oblonga*	N	N	unknown
TC03204413MX204	*P. arachnoides oblonga*	N	Y	unknown
TC0215712MC301	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC03192114MC401	*P. arachnoides oblonga*	N	N	*A. chabaudi*
TC02371415MC404	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC02291112MX402	*P. arachnoides arachnoides*	Y	Y	unknown
TC03264413MX202	*P. arachnoides oblonga*	N	N	unknown
TC0228812MX301	*P. arachnoides arachnoides*	Y	Y	unknown
TC0327515MC401	*P. arachnoides oblonga*	Y	N	*A. chabaudi*

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC03264413MX204	*P. arachnoides oblonga*	Y	Y	unknown
TC0225812MX201	*P. arachnoides arachnoides*	N	Y	unknown
TC02371415MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC02371415MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0238715MC401	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC0231812MC401	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC0230712MX301	*P. arachnoides arachnoides*	N	Y	unknown
TC0231812MX203	*P. arachnoides arachnoides*	Y	Y	unknown
TC02351415MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC02401415MX301	*P. arachnoides arachnoides*	Y	Y	unknown
TC0238715MC402	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC0231812MX302	*P. arachnoides arachnoides*	Y	Y	unknown
TC01921215MX402	*A. yniphora*	N	Y	unknown
TC01851215MX402	*A. yniphora*	N	Y	unknown
TC01894913MX203	*A. yniphora*	N	Y	unknown
TC018913MX202	*A. yniphora*	N	Y	unknown
TC01841215MX409	*A. yniphora*	N	Y	unknown
TC01931215MX303	*A. yniphora*	N	Y	unknown
TC01901215MX304	*A. yniphora*	N	N	unknown
TC01871215MX402	*A. yniphora*	N	Y	unknown
TC01894913MX201	*A. yniphora*	N	Y	unknown
TC01871215MX408	*A. yniphora*	N	N	unknown
TC01841215MX402	*A. yniphora*	N	N	unknown
TC01841215MX406	*A. yniphora*	N	Y	unknown
TC01781613MX201	*A. yniphora*	N	Y	unknown
TC01894913MX405	*A. yniphora*	N	Y	unknown
TC01871215MX401	*A. yniphora*	N	N	unknown
TC01901215MX403	*A. yniphora*	N	Y	unknown
TC01931215MX404	*A. yniphora*	N	N	unknown
TC01791215MX101	*A. yniphora*	N	Y	unknown
TC01921215MX403	*A. yniphora*	N	Y	unknown
TC01871215MX301	*A. yniphora*	N	Y	unknown
TC01771613MX401	*A. yniphora*	N	Y	unknown
TC01931215MX301	*A. yniphora*	N	Y	unknown
TC01881215MX401	*A. yniphora*	N	Y	unknown
TC01851215MX304	*A. yniphora*	N	Y	unknown
TC01781613MX406	*A. yniphora*	N	Y	unknown
TC01871215MX201	*A. yniphora*	N	Y	unknown
TC01861215MX301	*A. yniphora*	N	Y	unknown
TC01851215MX202	*A. yniphora*	N	Y	unknown

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC01921215MX409	*A. yniphora*	Y	N	unknown
TC019111215MX201	*A. yniphora*	N	N	unknown
TC01894913MX401	*A. yniphora*	N	Y	unknown
TC01931215MX401	*A. yniphora*	N	Y	unknown
TC01851215MX203	*A. yniphora*	N	N	unknown
TC01781613MX403	*A. yniphora*	N	N	unknown
TC01781613MX405	*A. yniphora*	N	Y	unknown
TC01881215MX403	*A. yniphora*	Y	Y	unknown
TC01851215MX302	*A. yniphora*	N	Y	unknown
TC01771613MX403	*A. yniphora*	N	N	unknown
TC01894913MX301	*A. yniphora*	N	Y	unknown
TC01791215MX201	*A. yniphora*	Y	Y	unknown
TC01901215MX302	*A. yniphora*	N	N	unknown
TC01791215MX204	*A. yniphora*	N	Y	unknown
TC01901215MX303	*A. yniphora*	Y	Y	unknown
TC01881215MX406	*A. yniphora*	N	N	unknown
TC01771613MX402	*A. yniphora*	N	N	unknown
TC01911215MX301	*A. yniphora*	N	Y	unknown
TC01851215MX403	*A. yniphora*	N	Y	unknown
TC01841215MX408	*A. yniphora*	N	Y	unknown
TC01791215MX404	*A. yniphora*	N	Y	unknown
TC01931215MX202	*A. yniphora*	N	Y	unknown
TC01921215MX401	*A. yniphora*	N	N	unknown
TC01791215MX402	*A. yniphora*	N	Y	unknown
TC01824913MX401	*A. yniphora*	N	Y	unknown
TC01824913MX402	*A. yniphora*	N	Y	unknown
TC01781613MX404	*A. yniphora*	N	Y	unknown
TC01881215MX405	*A. yniphora*	N	Y	unknown
TC01841215MX404	*A. yniphora*	N	Y	unknown
TC01791215MX403	*A. yniphora*	N	Y	unknown
TC01894913MX402	*A. yniphora*	N	Y	unknown
TC01931215MX201	*A. yniphora*	N	Y	unknown
TC01931215MX304	*A. yniphora*	N	Y	unknown
TC01901215MX401	*A. yniphora*	N	Y	unknown
TC01861215MX405	*A. yniphora*	N	Y	unknown
TC01871215MX202	*A. yniphora*	N	Y	unknown
TC01871215MX403	*A. yniphora*	N	Y	unknown
TC01831215MX306	*A. yniphora*	N	Y	unknown
TC01841215MX403	*A. yniphora*	N	N	unknown
TC01861215MX402	*A. yniphora*	N	Y	unknown

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC01911215MX203	*A. yniphora*	N	Y	unknown
TC01831215MX201	*A. yniphora*	N	Y	unknown
TC01861215MX403	*A. yniphora*	Y	Y	unknown
TC01831215MX303	*A. yniphora*	N	N	unknown
TC01861215MX408	*A. yniphora*	N	Y	unknown
TC01894913MX404	*A. yniphora*	N	Y	unknown
TC01841215MX407	*A. yniphora*	N	Y	unknown
TC01871215MX404	*A. yniphora*	N	Y	unknown
TC01841215MX401	*A. yniphora*	N	Y	unknown
TC01861215MX407	*A. yniphora*	N	Y	unknown
TC01921215MX406	*A. yniphora*	N	N	unknown
TC0176813MC401	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC01851215MX401	*A. yniphora*	N	Y	unknown
TC01861215MX404	*A. yniphora*	N	Y	unknown
TC04801213MC302	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC01811215MX403	*A. yniphora*	N	Y	unknown
TC01901215MX402	*A. yniphora*	N	Y	unknown
TC01881215MX301	*A. yniphora*	N	Y	unknown
TC01824913MX301	*A. yniphora*	N	Y	unknown
TC01851215MX201	*A. yniphora*	N	Y	unknown
TC04801213MC401	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC0175813MC401	*P. arachnoides arachnoides*	N	Y	*A. chabaudi*
TC01921215MX405A	*A. yniphora*	N	N	unknown
TC01824913MX302	*A. yniphora*	N	Y	unknown
TC01861215MX406	*A. yniphora*	N	Y	unknown
TC01911215MX204	*A. yniphora*	N	Y	unknown
TC01831215MX202	*A. yniphora*	N	Y	unknown
TC04801213MC303	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC01824913MX201	*A. yniphora*	N	Y	unknown
TC01861215MX401	*A. yniphora*	N	Y	unknown
TC01881215MX404	*A. yniphora*	N	Y	unknown
TC01871215MX407	*A. yniphora*	N	N	unknown
TC01831215MX302	*A. yniphora*	N	N	unknown
TC01791215MX405	*A. yniphora*	N	Y	unknown
TC01851215MX404	*A. yniphora*	N	Y	unknown
TC01811215MX402	*A. yniphora*	N	Y	unknown
TC01871215MX405	*A. yniphora*	N	Y	unknown
TC01901215MX405	*A. yniphora*	N	N	unknown
TC01931215MX406	*A. yniphora*	N	N	unknown
TC01901215MX301	*A. yniphora*	N	Y	unknown

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC01781613MX402	*A. yniphora*	N	N	unknown
TC01931215MX302	*A. yniphora*	N	N	unknown
TC01911215MX302	*A. yniphora*	N	N	unknown
TC01861215MX302	*A. yniphora*	N	Y	unknown
TC01811215MX401	*A. yniphora*	N	N	unknown
TC01894913MX403	*A. yniphora*	N	Y	unknown
TC01871215MX406	*A. yniphora*	N	Y	unknown
TC01921215MX410	*A. yniphora*	N	N	unknown
TC01831215MX305	*A. yniphora*	N	Y	unknown
TC01921215MX407	*A. yniphora*	N	Y	unknown
TC01831215MX301	*A. yniphora*	N	Y	unknown
TC01771613MX404	*A. yniphora*	N	Y	unknown
TC01931215MX403	*A. yniphora*	N	N	unknown
TC04801213MC301	*P. arachnoides oblonga*	N	Y	*A. chabaudi*
TC01811215MX405	*A. yniphora*	N	Y	unknown
TC01921215MX404	*A. yniphora*	N	N	unknown
TC01921215MX405B	*A. yniphora*	N	N	unknown
TC01931215MX402	*A. yniphora*	N	Y	unknown
TC01841215MX405	*A. yniphora*	N	N	unknown
TC01811215MX406	*A. yniphora*	N	N	unknown
TC01921215MX411	*A. yniphora*	N	N	unknown
TC01881215MX402	*A. yniphora*	N	Y	unknown
TC01901215MX404	*A. yniphora*	N	N	unknown
TC01791215MX401	*A. yniphora*	N	Y	unknown
TC01831215MX304	*A. yniphora*	N	N	unknown
TC01851215MX303	*A. yniphora*	N	Y	unknown
TC01911215MX206	*A. yniphora*	N	N	unknown
TC01931215MX405	*A. yniphora*	N	N	unknown
TC01824913MX303	*A. yniphora*	N	N	unknown
TC01811215MX404	*A. yniphora*	N	N	unknown
TC01831215MX203	*A. yniphora*	N	N	unknown
TC01911215MX205	*A. yniphora*	N	N	unknown
TC0175813MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC01791215MX205	*A. yniphora*	N	Y	unknown
TC01781613MX401	*A. yniphora*	N	N	unknown
TC0176813MC402	*P. arachnoides arachnoides*	Y	Y	*A. chabaudi*
TC01921215MX301	*A. yniphora*	N	Y	unknown
TC01791215MX203	*A. yniphora*	N	N	unknown
TC01791215MX202	*A. yniphora*	N	Y	unknown
TC01851215MX301	*A. yniphora*	N	Y	unknown

Sample ID	Host	*Borrelia*	*Rickettsia*	Morphological Tick ID
TC01824913MX403	*A. yniphora*	N	Y	unknown
TC01901215MX406	*A. yniphora*	N	Y	unknown
TC01911215MX202	*A. yniphora*	N	Y	unknown

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SELECTED GRANTS RECEIVED

Phan, A. 2018. Fellowship of Women in Science Travel Award.
Phan, A. 2018. American Society for Microbiology- Virginia Branch Travel Award.

SELECTED PUBLICATIONS

Benham, S.A., Gaff, H.D., Bement, Z.J., Blaise, C., Cummins, H.K., Ferrara, R., Moreno, J., Parker, E., **Phan, A.**, Rose, T., Azher, S., Price, D., Gauthier, D.T. 2020. Comparative population genetics of *Amblyomma maculatum* and *Amblyomma americanum* in the mid-Atlantic United States. Ticks and Tick-Borne Diseases. 12: 101600. https://doi.org/10.1016/j.ttbdis.2020.101600

Cumbie A., Heller, E., Bement, Z., **Phan, A.**, Walters, E., Hynes, W., and Gaff, H. 2021 Possible role of passerine birds in the spread and maintenance of *Borrelia burgdorferi* in *Ixodes* tick species in the mid-eastern United States. Ticks and Tick-Borne Diseases. 12: 101650. https://doi.org/10.1016/j.ttbdis.2021.101650