DNA barcoding of medically important mosquitoes and molecular detection of *Rickettsia felis* in the mosquitoes and the blood of domestic dogs and cats

by

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Abstract

Rickettsia felis, an obligate intracellular bacterium, is the causative agent of human flea-borne spotted fever. The recent surge of reports of this disease has raised the concern of a near-future outbreak as the list of vectors, reservoirs, and hosts of this pathogen are increasing with recent studies. Due to the close inhabitation of humans and the mammalian hosts, cats and dogs, and the ubiquitous distribution of the main vector, *Ctenocephalides felis*, it is not uncommon for companion animals and humans to get infected with *R. felis*. Mosquitoes have also been found to carry this pathogen in different countries. As a result, this pathogen retains a potential to disseminate among the human population. Mosquito species identification was required to study field-level mosquitoes for *R. felis*. This thesis first establishes and validates a highly sensitive DNA barcoding method for medically important mosquitoes (Chapter 2). Then, the presence of *R. felis* DNA in mosquitoes and in the blood of domestic cats and dogs in the USA were investigated (Chapters 3 and 4).

Field level mosquitoes were trapped in Auburn, Alabama, with mosquito trappers. A SYBR-qPCR system targeting the mitochondrial Cytochrome Oxidase C Subunit 1 (COI) was developed, optimized, and validated for the accurate determination of mosquito species. Furthermore, mosquitoes were tested for the presence of *R. felis* DNA using a *gltA*-based FRET-qPCR, a nested-qPCR targeting the *gltA* of *Rickettsia*, and a *R. felis* species-specific *BioB*-based qPCR system. Domestic cat blood samples from 752 cats from 43 states and domestic dog blood samples from 777 dogs from 45 states of the USA were tested for the presence of *R. felis* DNA with the same PCR systems. Following Sanger sequencing, the sequences were analyzed and were submitted to the GenBank. After assigning the accession numbers, phylogenetic trees were constructed.

The AU-COI PCR system was validated which show sensitivity of a single copy of DNA/10 µL (*Ae. japonicas, An. quadrimaculatus, Cx. nigripalpus, Cx. usquatissimus, Or. Alba, Ps. ferox, and Ur. sapphirina*) and 10 copies/10 µL (*An. punctipennis*). Eight mosquito species were successfully identified by morphology, published PCR from *Folmer et al. 1994*, and the AU-COI PCR system. In addition, seven mosquito species were correctly identified by both of two PCR systems, but not by the morphology. Furthermore, nine mosquito species were only accurately identified by the AU-COI PCR, not by the published one or by the morphology. In this study, the AU-COI DNA barcoding method detected 24 mosquito species out of 128 individual mosquitoes, which included two new *Culex* and three new *Psorophora* species in Alabama.

Anopheles punctipennis, Aedes vexans and *Uranotaenia sapphirina* were found positive for *R. felis*. Molecular techniques detected *R. felis* in 9% of the mosquito pools. Nine percent (5/57) of the mosquito pools, including *An. punctipennis* (3/6), *Ae. vexans* (1/4) and *Ur. sapphirina* (1/3), were positive by PCR, in each case with all three Rickettsia specific PCRs. One of the positive *An. punctipennis*, and the positive *Ur. sapphirina* pool contained only male mosquitoes. The 120-bp nucleotide sequences of the five mosquito pools positive in *R. felis*-specific *BioB*-based PCRs were identical to one another, and to that of *R. felis* URRWXCal2. There was only a single base pair difference amongst the 446-bp nucleotide sequences of the positive *gltA*-based PCRs which were 99.7–100% identical to recognized *R. felis* strains in GenBank.

Four *R. felis* positive cats were from different states: Kansas, California, New York, and Texas, whereas three *R. felis* positive dogs were from Texas and Georgia. Low copy numbers of *R. felis* DNA (around 100 copies/ml whole blood) were found in four cats (4/752, 0.53%) and three dogs (3/777, 0.39%).

The DNA barcoding of mosquitoes is unaffected by morphological variation between different stages of the life cycle. This technology precisely identifies even the cryptic mosquitoes which is vital for the accuracy of a surveillance program as it greatly reduces the burden on experienced taxonomists. Results indicated the presence of *R. felis* in mosquitoes and in domestic cats and dogs in the USA. There is still much to be understood about the vector and reservoir role of the wide range of arthropods that harbor *R. felis*. The growing reports of *R. felis* occurring in mosquito species around the world and the known role of mosquitoes in transmitting a wide range of important human and animal pathogens indicate the necessity of further studies to determine the role mosquitoes play in the epidemiology of infectious diseases. The low levels of infection in clinically ill animals are consistent with *R. felis* being an unlikely cause of disease in naturally infected dogs and cats.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ae.	*Aedes*
An.	*Anopheles*
ATP	Adenosine tri phosphate
BLAST	Basic Local Alignment Search Tool
COI	Cytochrome Oxidase C Subunit 1
Cs.	*Culiseta*
Cx.	*Culex*
Dei.	*Deinocerites*
ELISA	Enzyme Linked Immunosorbent Assay
*glt*A	Citrate Synthase
IFA	Immunofluorescence Assay
kb	Kilobase
Ma.	*Mansonia*
MIC	Minimum Inhibitory Concentration
MIF	Microimmunofluorescence
MLST	Multi Locus Sequence Typing
Mpf	Mating pair formation
Oc.	*Ochlerotatus*
*omp*A	Outer Membrane Protein A
*omp*B	Outer Membrane Protein B
PGFE	Pulsed Field Gel Electrophoresis

Ps.	*Psorophora*
R. felis	*Rickettsia felis*
RFLP	Restriction Fragment Length Polymorphism
RMSF	Rocky Mountain Spotted Fever
sca	Surface Cell Antigen
SFG	Spotted Fever Group
TCA	Tricarboxylic Acid
TG	Typhus Group
TOT	Transovarial transmission
Tx.	*Toxorhynchites*
Ur.	*Uranotaenia*
Wy.	*Wyeomyia*
XTC-2	*Xenopus laevis* carcass fibroblast cell line

Chapter 1. Literature Review

Mosquito

Potential role of mosquito as a vector

Mosquitoes (Diptera: *Culicidae*) play a crucial role among vectors of medical and veterinary importance, despite decades of extensive research efforts (Benelli, 2015). Mosquitoes are the most notorious vectors of infectious diseases among all the hematophagous arthropods. More than half of the global population is at risk of exposure to mosquito-borne diseases (Dieme et al., 2015). In general, bloodsuckers are not only troublesome because of their nuisance but also are dangerous due to their ability to transmit agents of diseases such as viruses, bacteria, fungi, and/or parasites. These organisms may cause serious ailments, even death (Mehlhorn, 2016). Transmission may occur during their oral uptake of blood or sucking lymph fluid when they insert their mouthparts into blood vessels (for example, mosquitoes).

Besides the widely known malaria burden, the dengue virus puts 3.9 billion people at risk in 128 countries. Both of these public health problems are related to mosquitoes. Zika virus outbreaks in the Americas and the Pacific attracted high public health attention while lymphatic filariasis remains one of the most important neglected tropical diseases (Bhatt et al., 2013; Petersen et al., 2016). All known bloodsuckers run their life cycles in their typical endemic regions successfully. They simultaneously expand their territory as a result of the enormously increasing globalization process through daily transportation of goods, persons, and animals from one end of the world to the other, where they are constantly supported by global warming. Mosquitoes are among the most effective of such hematophagus vectors to adapt to newer geological locations as well as novel environments.

Epidemics may be successfully blocked with the implementation of proper scientific strategies based upon research. Recent evidence shows that transmission of bluetongue epidemics of ruminants was blocked in central and northern Europe during 2006-2009 and the spread of the Chikungunya virus in Central Italy was successfully managed in 2007 (Kampen & Werner, 2010; Mehlhorn et al., 2009; Rezza et al., 2007). However, strong efforts to avoid the spread of hematophagous vectors and to block the transmission of pathogenic agents are always needed. Vectors were responsible for the spread of zoonotic diseases in one-quarter of pathogen outbreaks during the last century when most of the pandemics originated by zoonoses in the wild. Hence, the control of emerging and re-emerging vector-borne diseases constitutes one of the most important concerns of human health and global economies (Alcaide et al., 2009). As mosquitoes are the deadliest among all the vectors, they warrant more attention than any other arthropod vector.

Moreover, different human activities invading natural habitats and breeding sites such as unplanned urbanization, construction of dams, irrigation, along with the surge of international traveling and expansion of tourism are playing a role in the relocation of endemic mosquito-borne pathogens in newer geographical landscapes (Adilah-Amrannudin et al., 2018; Gratz, 1999; Jones et al., 2008). Therefore, there is a dire need for effective global mosquito surveillance and control programs to deal with the epidemiological risks of serious diseases. Precise identification of mosquitoes at their species level is crucial to develop optimized mosquito control strategies (Anoopkumar et al., 2019). However, comprehensive taxonomic knowledge of mosquitoes is far from complete although mosquitoes have been studied extensively for decades (Wang et al., 2012). Hence, mosquitoes get the attention of the most widely distributed public health control and disease prevention programs throughout the world. Field level mosquito research as well as

laboratory research, including use of animal models are important for studying different mosquito-borne diseases.

Medically important mosquitoes

Mosquitoes belong to the *Culicidae* family, which includes 42 genera consisting of around 3,560 reported species worldwide. Mosquitoes are found in every corner of the world except Antarctica. Among them, 190 species in 13 genera live in North America (Shahhosseini et al., 2020). All of these mosquitoes are not equally harmful. Mosquito species which transmit medically important pathogens are the matter of concern. Transmission can be mechanical or biological.

Mosquitoes are the most infamous arthropod vector in the world transmitting protozoa, bacteria, and viruses. Mosquitoes transmitting malaria, filaria, dirofilaria, and different viruses like Zika, Dengue, Chikungunya, Japanese encephalitis, Yellow fever, Rift Valley fever, West Nile, and Western Equine Encephalitis virus are most important. Along with these pathogens, mosquitoes transmit bacterial pathogens, such as, *Rickettsia* as well (Anoopkumar et al., 2019; Hernández-Triana et al., 2019).

Mosquitoes of *Aedes, Anopheles,* and *Culex* genera are mostly responsible for the transmission of major pathogens. *Ae. aegypti, Ae. albopictus, Ae. africanus, Ae. canadensis, Ae. sollicitns and Ae. vigilax* are important aedes species which transmit different arboviruses. *An. freeborni, An. gambiae, An. quadrimaculatus* play role in malaria transmission. *Cx. pipiens, Cx. tarsalis, Cx. quinquefasciatus* are important *Culex* mosquitoes to transmit arbovirus, fialria, and protist parasites (Benelli & Mehlhorn, 2018).

DNA barcoding

DNA barcoding is a tool for rapid species identification using a short fragment of DNA sequence (400-800 bp) from a specific gene or genes. It works in a similar fashion like the way a supermarket scanner identifies an item. The scanner uses the familiar black stripes of the UPC barcode to identify an item in its stock against its reference database. Similarly, DNA barcoding system compares an individual sequence to a reference library of such DNA sequences to uniquely identify the species of an organism. These DNA barcodes can identify an unknown species, parts of an organism, or catalog as many taxa as possible.

A good DNA barcode region should have low intra-specific and high inter-specific variability and possess conserved flanking sites for developing universal PCR primers. Different target regions of DNA are used to barcode different organismal groups, such as, internal transcribed spacer (ITS) rRNA for fungi (Schoch et al., 2012), Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) for plants (Plant & Group, 2009), 16S rRNA for prokaryotes, and 18S rRNA for microbial eukaryotes. The most popular DNA barcode region for animals and some protists is the mitochondrial cytochrome c oxidase I (COI or COX1) gene. *Folmer et al*. in 1994, first utilized this concept for phylogenetic analyses at the species levels and demonstrated it as a suitable discriminatory tool to differentiate metazoan invertebrates (Folmer et al., 1994). This COI region is called the “Folmer” or “Universal” region for DNA barcoding.

For barcoding, DNA is first extracted and amplified, and then the amplified product is sequenced using different sequencing methods, for example, Sanger sequencing or high-throughput next generation sequencing. Finally, the sequences are aligned by using bioinformatics analysis with reference libraries or databases (for example, Barcode of Life Data System, UNITE database, Diat.barcode database) containing DNA barcodes assigned to previously identified taxa.

The Basic Local Alignment Search Tool (BLAST) is commonly used to search and compare sequence reads of the sample to the sequences in reference databases to identify regions of similarity between sequences. A match of a sequence with a reference sequence diagnoses the species. DNA barcoding gives best result when it is used as a complement to traditional morphological methods (Pawlowski et al., 2018). As identification of mosquito species is important to study mosquito-borne diseases, and mosquitoes are likely to get damaged while handling field-level mosquito specimen, DNA barcoding is a useful method to identify mosquitoes at their species level.

What is unknown regarding *Rickettsia felis* in mosquitoes

Detection of a pathogen in field-collected specimens of a hematophagous arthropod needs to be critically assessed to determine its vector competence. Depending on the detection procedure and technique, it suggests the imbibement of a pathogen, but it does not confirm the ability of the pathogen to develop in that arthropod or whether the insect host serves as a biological vector. For example, a suspected vector-borne pathogen may be detected in mosquitoes by the processing of the complete mosquito or certain body parts of it, by using a microscopic, serologic or genetic approach. However, to be transmitted biologically, the ingested pathogen has to pass the midgut epithelium (i.e. to infect the mosquito), to disseminate, to replicate, or maturate, and to finally invade the different body organs of the female mosquito from where release and transmission ensue. Hence, the detection of the pathogen in the thorax or salivary gland of the mosquito generally establishes the vector competence of the mosquito for that pathogen (Hardy et al., 1983; Jupp, 1985).

For a mosquito to be vector-competent for a pathogen, it has to acquire and transmit a pathogen (Beerntsen et al., 2000). As a result of mutual adaptation of a hematophagous arthropod

and the pathogen, an arthropod species may be vector-competent for the pathogen, which features similar physiological and biochemical traits of the natural vector. This evolutionary adaptation can happen even in the absence of prior exposure of the ancestors of the arthropod to the pathogen. Moreover, the degree of vector competence can be modified as a result of spontaneous mutations. For example, it has been found that a certain mutant strain of the Chikungunya virus, which was responsible for the 2005–2006 epidemic on La Réunion Island, was able to transmit more efficiently than a non-mutant viral strain. This mutant strain was easily transmitted by *Ae. albopictus* rather than by *Ae. aegypti* (Tsetsarkin et al., 2007).

Vector competence varies among different populations of the same insect species under the same conditions (Weng et al., 1997). Different internal and external factors influence vector competence and transmission efficiency. Stressors, such as environmental temperature, larval density, food shortage, or exposure to insecticides influence vector competence. The surrounding temperature during the period of extrinsic incubation of the pathogen influences transmission efficiency when the pathogen remains in an infected arthropod (Muturi et al., 2012; Muturi & Alto, 2011; Turell, 1993). On the other hand, vector capacity depends on vector and blood host density, blood host preference, biting rate, duration of the extrinsic incubation period of the pathogen, probability of the vector to become infective, and duration of its infective life (Reisen, 1989). Thus, a mosquito species may be vector-competent without playing the role of a vector. Vector capacity defines the role of a vector-competent arthropod in a certain environment.

R. felis has been detected in numerous field-collected mosquito species and other potential hematophagous arthropods. Vector competence of mosquitoes for *R. felis* can only be demonstrated with experimental studies. Experimental demonstration of pathogen release to a vertebrate host or into a buffer solution, on a membrane or a cotton wool pad, while the mosquito

is feeding, is considered as valid proof of vector competence. *R. felis* may act differently in different environments in different indigenous mosquito species. Though *R. felis* has long been known to be present in fleas, cats, and dogs, the recent surge of case reports of flea-borne rickettsioses around the world has revealed a knowledge gap regarding this pathogen. Moreover, it is being found in newer species of mosquitoes in different geographical locations. It has already been detected in different invasive and potential vector mosquitoes, such as *Anopheles gambiae* (Dieme et al., 2015), *Culex Pipiens, Aedes albopictus, Anopheles sinensis* (Zhang, Lu, Li, et al., 2019), *Aedes aegypti, Culex quinquefasciatus* (Valentine et al., 2021), *Anopheles punctipennis*, and *Aedes vexans* (Barua et al., 2020). Among them, *Anopheles gambiae* is the primary malarial vector in sub-Saharan Africa. This mosquito species could be responsible for a major outbreak of *R. felis* in the near future.

Rickettsia

Introduction to *Rickettsia* spp.

"*Rickettsia*" has long been used as a generic term for many obligate intracellular small bacteria expressing few phenotypic characters that could not be cultivated conventionally and were not otherwise identified (Raoult et al., 2005). However, the study of gene and genome evolution with molecular techniques has significantly changed the taxonomic inferences and reclassified the *Rickettsiales* over the last four decades (Birtles et al., 1995; Brenner et al., 1993; Dumler et al., 2001; Neimark et al., 2001; Roux, Bergoin, et al., 1997; Roux & Raoult, 1995; Stothard & Fuerst, 1995; Tamura et al., 1995; Weisburg et al., 1989). Currently, the term "*rickettsia*" applies only to arthropod-borne α-Proteobacteriae, which belongs to the genus *Rickettsia* within the family *Rickettsiaceae* in the order *Rickettsiales*. The genus is named after Howard Taylor Ricketts who

described the transmission of Rocky Mountain spotted fever (RMSF) in 1906 (Ricketts H T, 1906). Despite the early recognition of the diseases caused by *Rickettsia* and the pathogen itself, many of the species are labeled as agents causing emerging, re-emerging, and/or neglected infections (Raoult & Roux, 1997). The *Rickettsia* genus is currently made of 26 recognized species (Fang et al., 2017), but many isolates await taxonomic classification (El Karkouri et al., 2016). Though more knowledge is available now, there is still knowledge gap regarding *Rickettsia*. It needs more research and more knowledge to monitor emerging species, perform accurate and prompt diagnoses, develop better methods to study the organism, and apply appropriate techniques to study their pathological effects on humans and animals (Wallménius, 2016).

Rickettsiae cause the oldest known arthropod-borne diseases called rickettsioses. Evidence of rickettsioses predates the 16th century, and was suggested in ancient Athens during the 5th century BC (Raoult & Roux, 1997). Most of these bacteria are vectored by ticks, lice, fleas, or mites, which may also serve as reservoirs (Azad & Beard, 1998; Kohls, 1947). Some rickettsioses have specific geographic distributions, depending on the distribution of their vectors. *Rickettsiae* are potential human pathogens, found in arthropods that are capable of biting humans. *Rickettsia* species diverged into three phylogenetic groups: the spotted fever group (SFG) associated with ticks, fleas, and mites, the typhus group (TG) associated with body lice, and rat fleas, and the ancestral group (AG) associated with ticks (Raoult & Roux, 1997; Stothard et al., 1994). *Rickettsia felis* belongs to the spotted fever group which is vectored by cat fleas and has a worldwide geographical distribution.

Characteristics of *Rickettsia felis*

Rickettsia felis, the causative agent of flea-borne spotted fever (also called cat flea typhus) is a small (0.8–2 m in length and 0.3–0.5 m in diameter), rod-shaped, Gram-negative, fastidious,

obligate intracellular bacilli. The cytoplasm of Rickettsia contains ribosomes and strands of DNA. It has a tri-laminar cell wall comprised of a bilayer inner membrane, a peptidoglycan layer, and a bilayer outer membrane. It remains surrounded by electron-lucent clear spaces inside the host cells (Bouyer et al., 2001; Ormsbee, 1969; Weiss, 1982). R. felis polymerizes actin within the cytoplasm of infected cells hence it migrates into the nucleus **(Figure 1**) (Ogata, Robert, et al., 2005). It is not stained by the Gram method but retains basic fuchsin when stained by the Gimenez method (Giménez, 1964). Microscopically, it exhibits two types of pili. The longer pili are sex pili that play the role of the mating pair formation (Mpf) apparatus during conjugation. The shorter pili help in the pathogenicity of the bacteria by providing means of attachment to the cell surface. *R. felis* possesses two plasmids (pRF and pRFδ) (Gillespie et al., 2007) and its chromosome is longer (1,485 kb) than other previously sequenced *rickettsiae*. The longer plasmid plays a role in conjugative plasmid transfer. Interestingly, plasmid content can vary among different isolates of *R. felis* (Baldridge et al., 2008). *R. felis* contains several copies of Ankyrin-repeat genes and tetratricopeptide encoding gene. Moreover, being intracellular bacteria, it possesses rare toxin-antitoxin system encoding genes (Ogata, Robert, et al., 2005).

The culture of *R. felis* is challenging. *R. felis* does not grow on conventional agar media or in broth. It can be grown in XTC-2 (adult *Xenopus laevis* carcass fibroblast cell line) cells at 28° C and Vero cells at 28 and 32° C. *R. felis* induces cytopathic foci in XTC-2 at 9 days and plaque formation in Vero cells at 18 days (La Scola et al., 2002). This organism is passed transstadially and transovarially, and infection in the cat flea has been observed in the midgut, tracheal matrix, muscle, hypodermis, ovaries, and testes (Bouyer et al., 2001).

Rickettsioses have been named for their geographical distribution, or their vector and/or clinical features, or their epidemiological feature (Raoult et al., 2005). The agent of flea-borne

spotted fever was first described as *R. ctenocephali* in 1918 (Wolbach, 1919), but now it is named *R. felis* after the discovery and origin of the bacterium in the cat flea (*Ctenocephalides felis*). The cat flea is a parasite of the cat (*Felis domesticus*) and opossum (*Didelphis marsupialis*) (Adams et al., 1990; Higgins et al., 1996; Matthewman et al., 1997).

Taxonomy and phylogeny of *R. felis*

As *rickettsia* is an obligate and fastidious intracellular bacterium and expresses very few phenotypic characteristics, it was challenging to classify this bacteria taxonomically. In fact, the strict intracellular nature of *R. felis* hindered progress in the detailed characterization of its phenotypic diversity (Ogata, Robert, et al., 2005). Conventional phenotypic methods which are used to classify axenically cultivable bacteria failed to classify *Rickettsiales* taxonomically. The development of PCR and nucleotide sequencing has considerably modified the taxonomic classification of bacteria. In particular, the study of 16S rRNA or rDNA has helped to resolve the enigmatic classification of intracellular bacteria (Dumler et al., 2001; Tamura et al., 1995).

Despite the usefulness of Pulsed-field gel electrophoresis (PFGE) in differentiating *rickettsiae,* it has some limitations due to the absence of any database which allows comparison of PFGE profiles. Thus, it lacks reproducibility. Over the last two decades, several genes have been used to rapidly and reliably differentiate members of the genus *Rickettsia.* These included the genes encoding 16S rRNA (16S rDNA), citrate synthase (*gltA*), the 17-kDa common antigen, surface-exposed, and high-molecular-weight antigenic proteins of the *sca* family (*ompA, ompB, sca*4, *sca*1, and *sca*2) either by analysis of PCR–RFLP or by direct sequence determination (B. E. Anderson & Tzianabos, 1989; Fournier et al., 1998; Ngwamidiba et al., 2005, 2006; Roux, Rydkina, et al., 1997; Roux & Raoult, 1995, 2000; Sekeyova et al., 2001). Later, to classify the bacterial isolates as *rickettsiae* at genus, group, and species levels, genetic criteria based on a multi-

locus sequence typing (MLST) method was developed (Fournier et al., 2003). The MLST-based criteria used sequences of the 16S rDNA, *gltA*, *ompA*, *ompB*, and *sca*4 genes. Finally, a polyphasic approach incorporating phenotypic, genotypic, and phylogenetic criteria enabled the classification of rickettsial isolates of uncertain taxonomic rank (Ogata et al., 2002).

Initially, phylogenetic analysis of *rickettsiae* was based on the conventional methods which were used to study other prokaryotes: comparison of morphological, antigenic, and metabolic features. However, phylogenetic relationships based on these criteria were unreliable for *rickettsiae*. The post-genomic phenotypic analysis provided better insight in this regard. Studies of transcriptomics and proteomics helped in associating the *in vivo* phenotypes of these bacteria to their genomic features (Ogata, Renesto, et al., 2005). Genome sequencing provided evidence of conjugative plasmid in *R. felis*. Furthermore, genomic findings identified two types of pili, β-lactamase activity, hemolytic activity, and intracellular motility in *R. felis*. Thus, complete genome sequencing proved to be potent in identifying unrecognized phenotypic traits. The first gene to be used for phylogenic purposes was the 16S rDNA (Roux & Raoult, 1995; Stothard et al., 1994). Subsequently, phylogenic studies were inferred from sequences of *gltA* (Roux, Rydkina, et al., 1997), the gene encoding the 17-kDa protein (B. E. Anderson & Tzianabos, 1989), and genes from the autotransporter family *sca*: *ompA* (Fournier et al., 1998), *ompB* (Roux & Raoult, 2000), *sca*4 (Sekeyova et al., 2001), *sca*1 (Ngwamidiba et al., 2006), and *sca*2 (Ngwamidiba et al., 2005).

The genus *Rickettsia* evolved from a common ancestor of *Rickettsiales* approximately 150 million years ago. Before that, several transitions occurred through which the presumably free-living ancestor evolved into an obligate intracellular organism about 775-525 million years ago, and then approximately 525-425 million years ago it further evolved to *Rickettsiales*, primarily infecting arthropod lineages (Diop et al., 2019; Weinert et al., 2009). *Rickettsiae* can infect humans

or animals mostly through arthropod bites (El Karkouri et al., 2016). Comparative genomics revealed that *rickettsiae* underwent a genome reductive evolution which occurred through a progressive pseudogenization and losing of selected biosynthetic pathway components (Fournier et al., 2009; Sakharkar et al., 2004; Walker & Yu, 2005; Wolf & Koonin, 2013). Genome sequence analyses reveal that *rickettsiae* are deficient in genes for encoding enzymes to metabolize sugar. However, genes for encoding enzymes of the tricarboxylic acid (TCA) cycle are present in the rickettsial genomes. *Rickettsiae* do not produce pyruvate but they utilize pyruvate by acquiring it from the host cell cytoplasm and they convert it into acetyl-CoA by pyruvate dehydrogenase. This suggests that *rickettsiae* can synthesize their own ATP. *Rickettsiae* are hypothesized to transport ATP from the host cell cytosol into *rickettsiae* during the early infection, when ATP is sufficient in the host cytosol and to synthesize ATP through the TCA cycle when the host cytosol ATP is exhausted. *Rickettsiae* generally lack genes for the synthesis and conversion of most amino acids in their genomes. They possess several genes for some amino acid biogenesis pathways. However, these pathways are usually incomplete because the downstream genes for these amino acid biosynthetic pathways are absent in rickettsial genomes. Rickettsial genomes have no genes for encoding enzymes for *de novo* synthesis of nucleotides. Thus, they probably import the nucleoside monophosphates from the host cell (Andersson et al., 1998; McLeod et al., 2004; Ogata et al., 2001; Walker & Yu, 2005). Thus reductive evolution explains the strict intracellular lifestyle of *rickettsiae*, where some metabolic pathways, such as genes encoding enzymes for sugar metabolism, lipid biosynthesis, nucleotide synthesis, and amino acid synthesis are progressively lost (Diop et al., 2019). To compensate for this impairment, host cells provide the missing metabolites (El Karkouri et al., 2016). This may explain why these bacteria have not been cultivated outside of eukaryotic cells (Walker & Yu, 2005).

Hosts and vectors of *R. felis*

Rickettsial organisms have been found in all continents except Antarctica. Different rickettsial species are predominant over different regions due to different climatic conditions and vector and natural host constraints. However, *Rickettsia felis* is globally distributed. The reservoir-vector dynamics of *R. felis* is a bit enigmatic and more complicated than other *rickettsiales*. In a classical "reservoir-vector" epidemiological scheme the reservoir host harbors and multiplies the pathogen, provides nourishment and shelter, and the vector (usually an arthropod) carries and transmits the pathogen into another living organism. Since active studies are going on the natural reservoirs and vectors of *R. felis*, the list is still expanding. (Angelakis et al., 2016). Until now, 39 species of arthropods have been found associated with *R. felis.*

The cat and dog fleas, *Ctenocephalides felis and Ctenocephalides canis* respectively, have been implicated as the natural vectors of *R. felis.* Both horizontal and vertical transmission make this flea a potential reservoir for the bacterium (Hirunkanokpun et al., 2011; Parola, 2011; Thepparit et al., 2013). Cats and dogs are the main hosts for these fleas and thus they facilitate the transmission of *R. felis* by amplifying the flea population. Along with cats and dogs, opossums are found most heavily infested with cat fleas in the United States (Azad et al., 1997). Apart from fleas, other arthropods such as mites (Y. J. Choi et al., 2007; De Castro Jacinavicius et al., 2019), lice (Behar et al., 2010), ticks (Ishikura et al., 2003) and recently mosquitoes (Barua et al., 2020) have also been found as potential vectors for this bacteria.

R. felis has been found in a total of 13 species of fleas, eight species of ticks, and one species of louse. It has also been found in five genera of mosquitoes. The important arthropods and their vertebrate hosts are enlisted in **Table 1**. Vertebrate infection may occur by flea bite during blood-feeding or fecal contamination of excoriations. Moreover, inhalation of small infected

particles (such as larvae and eggs of booklouse) may transmit the pathogen from booklice to vertebrates (Martinez et al., 2021a).

Reports of *R. felis* in mosquitoes have potentially changed our understanding of rickettsial vectors and transmission to human hosts as mosquitoes are known vectors for many diseases. The maintenance of rickettsiosis caused by *R. felis* occurs by transovarial transmission (Zavala-Velázquez et al., 2000). Hence, fleas may acquire *R. felis* from rickettsemic hosts and then pass on the infection to their progeny. *R. felis* infection does not exert lethal effects on the fleas. Even massive infection of flea midgut does not affect the feeding behavior and survival of the infected fleas (Azad et al., 1997). Rather the wide dissemination of the bacteria within the flea host suggests a close association between *R. felis* and the flea (Reif & Macaluso, 2009).

Epidemiology of *R. felis*

R. felis is a member species of the Spotted Fever Group that is transmitted by fleas and some other arthropod vectors and causes the zoonotic rickettsiosis called flea-borne spotted fever or cat flea typhus. Humans can be infected following bites by an infected arthropod (Azad et al., 1997). Clinical signs of the illness are similar to those of murine typhus and other febrile illnesses such as dengue and malaria. Moreover, the detection of *R. felis* was not easy for its being an obligate intracellular bacterium. As a result, *R. felis* infection in humans has been underestimated for a long time despite its being one of the oldest arthropod-borne zoonotic diseases. With the advent of sensitive and delicate molecular detection techniques, *R. felis* has been recovered from different arthropods which infest different mammals. These ectoparasites have been recovered from different vertebrate animals (**Table 1**). The presence of *R. felis* in a diverse range of invertebrate vectors and mammalian hosts represents a potential risk for public health hazard.

Vectors responsible for the transmission of *R. felis* have a worldwide distribution and infestation with these vectors is very common. As a consequence, flea-borne spotted fever may occur around the globe. *R. felis* can be found in most of the human populations where domestic animals are kept as pets. Co-migration of humans and domestic animals harboring *C. felis* played a role in the worldwide distribution of *R. felis.* The first human case of *R. felis* infection was reported in 1994 in Texas, USA (Schriefer et al., 1994).

In the decades since 1994, an increasing number of human case reports with *R. felis* infection through molecular or serologic detection techniques have been reported. Three case reports of *R. felis* infection were reported from Southern Mexico in 2000 (Zavala-Velázquez et al., 2000). In following years, human infection cases with *R. felis* increased rapidly along with the fast-growing reports of *R. felis* in arthropod hosts worldwide (Pérez-Osorio et al., 2008). More recently, *R. felis* was found as the most common bacterial cause of fever in patients in tropical countries in Africa after malaria. It has also been detected in tropical and subtropical areas of North and South America, Asia, and Oceania (Angelakis, 2016). Till today, *R. felis* has been reported molecularly in arthropod vectors in more than 40 countries spanning over five continents (Abdad et al., 2011; Reif & Macaluso, 2009) (**Table 1**).

As *R. felis* is not uncommon in cat fleas (Ogata, Robert, et al., 2005), and cats are kept as pets, humans are likely to get infected with this bacteria. Human cases of *R. felis* infection are being increasingly reported worldwide. The first human case was reported in Texas, USA. Since then human cases of *R. felis* infection have been reported from different countries (**Table 2**).

Clinical features of flea-borne spotted fever

Rickettsia felis infection causes cat flea typhus in humans which manifests as an undifferentiated febrile illness. It is very common to misdiagnose flea-borne rickettsioses due to

their nonspecific symptoms and signs similar to other febrile diseases. Consequently, such infections often go unrecognized by physicians. The differential diagnosis can be broad for flea-borne rickettsioses. This undifferentiated fever can include common bacterial infections (such as pneumonia, endocarditis, urinary tract infections, and meningitis), less frequent bacterial infections (such as leptospirosis, secondary syphilis, rat-bite fever, and disseminated gonorrhea), different viral diseases, and mononucleosis syndromes (i.e., Epstein-Bar virus, cytomegalovirus) (Martinez et al., 2021b). Therefore, it requires a high index of suspicion and sound knowledge of the clinical features and epidemiology of cat flea typhus for recognizing illness, establishing a confirmatory diagnosis, and initiating timely treatment.

Patients from tropical countries present with fever constantly. Fever, rash, and headache are considered to be the major triad of rickettsial disease. However, myalgia, arthralgia, cough, chest pain, malaise, nausea, loss of appetite, and macular or popular rash have been reported along with fever in different countries (Angelakis et al., 2012; Y. J. Choi et al., 2005; Edouard et al., 2014; Parola, 2011; Parola et al., 2003; Richards et al., 2010; Socolovschi et al., 2010; K.-H. Tsai et al., 2008). The end effects of the disease pathophysiology can manifest as pulmonary edema and other respiratory complications, gastrointestinal symptoms, meningitis, encephalitis, and even death. Eschar has been noted in some cases too (Martinez et al., 2021b). Severe manifestations of this disease have been reported in three patients in Mexico and two patients in Sweden, who had features of meningitis, photophobia, and loss of hearing (Lindblom et al., 2010; Zavala-Velázquez et al., 2000). One patient presented with pulmonary compromise (J. Zavala-Velazquez et al., 2006) and another two had severe respiratory insufficiency in Mexico (Zavala-Castro et al., 2009). Two deaths have been reported from meningoencephalitis following *R. felis* infection in Indonesia (Mawuntu et al., 2020).

Diagnosis of *R. felis* infection

The priority of proper management of any infectious disease is to establish a confirmatory diagnosis and initiate timely therapy. Following clinical assessments, laboratory investigations are conducted to detect the key evidence of the infection. As flea-borne spotted fever present with non-specific sign-symptoms of febrile illness, it is very difficult to diagnose this infection with clinical eyes only. However, knowledge of epidemiology and demographic characteristics of a patient helps the physician to include *R. felis* infection as a differential diagnosis. Flea-borne rickettsioses still lack rapid and accurate diagnostic tools for confirmatory diagnosis.

Abnormalities in basic hematological and biochemical laboratory findings, such as elevated hepatic transaminases and thrombocytopenia raise the concern of infection but they cannot exclude ambiguity as many other community-acquired and vector-borne infectious diseases give similar findings. Serology is the most popular laboratory diagnostic procedure for rickettsial diseases with different approaches. The Weil–Felix test, latex agglutination, and complement fixation methods are the old standard approaches for diagnosis of rickettsial infections. These are neither sensitive nor specific for the diagnosis, although Weil–Felix test is still used in some developing countries (Hechemy et al., 1979). These methods were supplanted by the indirect immunofluorescence assay (IFA), microimmunofluorescence (MIF), and enzyme-linked immunosorbent assays (ELISA). These tools are based on the detection of anti-rickettsial antibodies in a patient's blood. However, IFA gives inter- and intra-observer variation in endpoint titer interpretation. ELISA does not give a perfect endpoint titer when performed as a standard clinical diagnostic test (Lokida et al., 2020; Phetsouvanh et al., 2013). However, these serologic tools are unable to give a species-specific diagnosis due to cross-reacting antibodies formed as a result of infection by different *rickettsiae*. Moreover, these serologic techniques are not suitable

for rapid and early detection of the active infection because it needs 7-14 days to develop antibodies and then be detected by these antibody detection tools. These are rather retrospective and require demonstration of seroconversion or a 4-fold increase in antibody titer from acute and convalescent-phase specimens (Fang & Raoult, 2003; Martinez et al., 2021b).

Purported *R. felis* infections have been detected rapidly, successfully, and frequently in blood and biopsy or swab from skin lesion with different molecular techniques through PCR amplification and sequencing. Conventional PCR, qPCR, nested PCR, and isothermal amplification have been used targeting different regions of *R. felis* DNA to detect the pathogen in infected arthropod vectors, mammalian hosts, and human patients. Molecular approaches produce sensitive, specific and reproducible results for the diagnosis of *R. felis* infection (Brouqui et al., 2007; Parola, 2011). Over the last two decades, several genes have been used to rapidly and reliably differentiate members of the genus *Rickettsia.* These included the genes encoding 16S rRNA (16S rDNA), citrate synthase (*glt*A), the 17-kDa common antigen, high-molecular-weight antigenic surface cell antigen (*sca* family) proteins (*sca*1, *sca*2 and *sca*4), outer membrane protein A (*omp*A), outer membrane protein B (*omp*B), and biotin synthase (*BioB*). Amplification of these targets with different PCR platforms followed by sequencing confidently confirms the presence of the organism in the patient and field samples (B. E. Anderson & Tzianabos, 1989; M. Anderson et al., 2019; Fournier et al., 1998; Kato et al., 2013; Luce-Fedrow et al., 2015; Ngwamidiba et al., 2005, 2006; Prakash et al., 2009, 2012; Renvoisé et al., 2012; Roux, Rydkina, et al., 1997; Roux & Raoult, 1995, 2000; Sekeyova et al., 2001; Watthanaworawit et al., 2013; Zhang et al., 2014; Zhang, Lu, Kelly, et al., 2019)

Treatment of flea-borne spotted fever

Although proper and timely treatment of flea-borne rickettsioses leads to complete recovery, failure to treat accordingly can lead to prolonged illness. It is suggested to start empiric treatment for undifferentiated febrile illness rather than waiting for the diagnostic results. Prompt empiric therapy with an effective medication cuts down the number of physician visits and duration of hospitalization. Doxycycline, rifampin, fluoroquinolones (Ives et al., 2001), and telithromycin were found to be the most effective antibiotics *in vitro* against all strains of *Rickettsiae* (Gudiol et al., 1989; Raoult et al., 1986; Rolain et al., 1998). Different antimicrobials showed a range of variations in the Minimum inhibitory concentrations (MIC) (µg/mL). They (MICs in µg/mL) ranged from 0.06 to 0.125 for doxycycline, from 0.06 to 0.25 for rifampin, and from 0.5 to 1 for ciprofloxacin for *R. felis*. However, doxycycline is the treatment of choice. The recommended dose of doxycycline is 100 mg twice per day for all spotted fever rickettsioses (Purvis & Edwards, 2000; Raoult & Roux, 1997). If the illness is severe, a one-time 200 mg loading dose is administered. Other alternatives should be administered if the patient is allergic to doxycycline. The duration of therapy depends on the clinical response of the patient. However, a regimen of doxycycline for 7-14 days has shown effective to treat flea-borne rickettsioses (Brouqui et al., 2007). On the other hand, β-lactams, sulfonamides, such as trimethoprim-sulfamethoxazole have been found ineffective in treating this disease (Rolain et al., 1998, 2002). Children and pregnant patients need special attention while treating the infection. The preferred treatment for pregnant patients still remains doxycycline (Martinez et al., 2021b). Along with the medication, the patient needs proper supportive care, especially if develops complications.

Zoonotic potential of *R. felis*

Public health concerns of emerging *R. felis*

Flea-borne spotted fever is an emerging infectious zoonotic disease. It was considered rare even two decades ago. However, it is now one of the most widespread zoonotic pathogens in Africa and is extremely frequent in some regions. It is being reported with an increasing trend over a wide geographical area of the world.

In the case of vector-borne diseases, ecological changes which lead to an increase in vector population densities, play an important role in the emergence of the diseases. The geographical spread of the etiologic agent of a vector-borne disease into a new area starts with the introduction of the pathogen by travelers from an endemic region. However, if the new area does not have suitable vectors the disease does not spread, rather it remains as a medical problem for the individual and the physician. Even the presence of a suitable vector is not enough to ensure the establishment of the transmission of the disease in the new area. Climate, the density of human population and their status of immunity, the density of a potential vector population(s) and, mostly, presence of a suitable reservoir or host influence whether transmission can be established (Gratz, 1999; Wilson, 2005). Environmental changes, such as global warming and deforestation, irrigation, excessive agriculture, encroachment of human habitation, destruction of niche environments, increase in transport and communication, increased movement of humans and animals- all have prominently increased over the last few decades. These influential domains are creating new opportunities for the dispersion and establishment of novel infectious diseases (Soto, 2009).

Changes in ambient temperature and climate cause local vector populations to migrate to more favorable climates and alter their life cycle duration to cope with the changes. Feeding

activity, reproduction, and mortality rates of arthropod vectors, such as mosquitoes are highly sensitive to even slight changes in temperature. Both reservoirs and vectors for *R. felis* are universal in distribution. Moreover, many of the hosts of the vectors are kept as pet animals. Along with that, *R. felis* has a long list of vectors. These vectors, especially mosquitoes and cat fleas are ubiquitous in our surrounding environments. As a result, *R. felis* has all the essential components in its favor to spread and emerge as a potential public health concern. Over the last decade, reports of new cases of rickettsioses have significantly increased in the malaria-endemic regions of Africa along with newer malaria-prone zones of the world. As mosquitoes act as a vector for *R. felis*, global warming may be a cause of the recent surge of rickettsioses where mosquito-borne diseases are endemic (Zell, 2004).

Modern sophisticated and sensitive diagnostic tools facilitate to accurate and rapid detection of diseases and therefore enable more accurate reporting. Flea-borne spotted fever has overlapping signs and symptoms with other febrile diseases. It was challenging to culture in conventional methods as it is fastidious and strictly intracellular. Even serology was not conclusive as *Rickettsia* has many species and cross-reacting antibodies decrease the specificity of the test. These challenges were overcome with improvements of modern, sensitive molecular diagnostic tools. The use of these methods has facilitated detection, and enabled development of more robust tools for diagnosis of *R. felis* infection (Wallménius, 2016).

Rickettsioses due to *R. felis* infection is one of the main causes of non-malarial fever. There lies a significant correlation between *R. felis* and malaria concerning geographic distribution and seasonality. The vectors- fleas and mosquitoes play an important role in such a correlation (Mediannikov et al., 2013). It has been found that up to 15% of febrile patients in malaria-endemic

regions in sub-Saharan Africa, in particular in Senegal and Kenya suffer from cat flea typhus (Ehounoud et al., 2017).

Flea-borne spotted fever will be the next outbreak?

The vector-pathogen relationships of the rickettsioses have been well studied over the last century through intensive studies of Rocky Mountain Spotted Fever (RMSE), murine typhus, and epidemic typhus, following Howard Taylor Ricketts's significant work on RMSF and its tick vectors. However, the flea-borne spotted fever and its causative agent Rickettsia felis yet retain knowledge gaps.

As rickettsia-like agents are common in arthropods, coeval adaptive radiation might have taken place in rickettsia over tens of millions of years with arthropod speciation. The relationship between *rickettsiales* and invertebrates is both unique and diverse. The vector competence for a vector-borne organism comprises of several factors: a minimum infectious dose to initiate an infection in the vector (Burgdorfer et al., 1966); a generalized infection of virtually every tissue of the vector; a principal tissue reservoir such as the salivary gland or hindgut (Wolbach et al., 1922); the phenomenon of reactivation (Spencer & Parker, 1923); a salivarian mode of inoculation; and capacity for transovarial transmission. Vector fitness, vector longevity, nature of vector specificity, and host range are largely influenced by the agents themselves. Although there is a general paradigm for the vector aspects of rickettsial life cycles like the piroplasms have within ticks or malarial parasites within diptera, variations exist for different species of *rickettsiae*.

Diverse fleas, especially *Ctenocephalides felis*, were noted to harbor *Rickettsiae* (Wedincamp & Foil, 2002). *R. felis* was found to be stably maintained in fleas by transovarial transmission (TOT) for up to 12 generations without the benefit of an infected host. Thus a mammalian reservoir may not be essential for its perpetuation (Wedincamp & Foil, 2002).

Moreover, during co-feeding and mating, *R. felis* is horizontally transmitted between cat fleas. This combination of vertical and horizontal transmission contributes to the maintenance and enhanced dissemination of *R. felis* (Hirunkanokpun et al., 2011).

Mosquitoes are increasingly being reported to carry *R. felis* in different geographical regions of the world which favors the potential of mosquitoes to be successful for *R. felis*. Moreover, different invasive mosquito species are being found infected with *R. felis*. In sub-Saharan Africa where malaria is common, *R. felis* has already being considered a common cause of fever and an emerging pathogen. The presence of *R. felis* in a wide range of arthropods confers its wide adaptability in its vectors, reservoirs, and hosts to become the causative agent of a near-future outbreak.

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Table 1. Potential invertebrate and vertebrate hosts of *R. felis* with the country of origin

Location	Invertebrate host	Vertebrate host	Reference
Albania, Argentina, Australia, Brazil, Canada, Chile, China, Columbia, Costa Rica, Democratic Republic of Congo, Ethiopia, France, Gabon, Germany, Guatemala, Hungary, Israel, Italy, Kenya, Laos, Lebanon, Malaysia, Mexico, Netherlands, New Zealand, Panama, Peru, Spain, Taiwan, Tanzania, Tunisia, Thailand, UK, USA, Uruguay, West Indies	*Ctenocephalides felis*	Cat, dog, rodents, monkey, opossum	(America, 2007; Bauer et al., 2006; Bitam et al., 2006; Blair et al., 2004; Blanco et al., 2006; Gilles, Just, Silaghi, Pradel, Lengauer, et al., 2008; Gilles, Just, Silaghi, Pradel, Passos, et al., 2008; Horta et al., 2006; Hsu et al., 2011; Isozumi, 2012; Jiang et al., 2013; Kamrani et al., 2008; Kelly et al., 2004; Kenny et al., 2003; Kumsa et al., 2015; Nava et al., 2008; Oliveira et al., 2008; Parola et al., 2003; Ramírez-Hernández et al., 2013; Rolain et al., 2003, 2005; Schloderer et al., 2006; Silaghi et al., 2012; Tay et al., 2014; Tijsse-Klasen et al., 2011; Troyo et al., 2012; K. H. Tsai et al., 2009; Venzal et al., 2006; Williams et al., 1992; Zaremba & Smoleński, 2000; J. E. Zavala-Velazquez et al., 2002)
Algeria, Brazil, Columbia, France, Kenya, Laos, Spain, Thailand, Uruguay	*Ctenocephalides canis*	Dog, cat	(Bitam et al., 2006; Blanco et al., 2006; Gilles, Just, Silaghi, Pradel, Lengauer, et al., 2008; Horta et al., 2006; Jiang et al., 2013; Ramírez-Hernández et al., 2013; Tijsse-Klasen et al., 2011; Venzal et al., 2006)
USA	*Anomiopsyllus nudata*	Rodents	(Stevenson et al., 2005)
Algeria, France, Germany, Spain	*Archaeopsylla erinacei*	Hedgehog, dog, cat	(Bitam et al., 2006; Blanco et al., 2006; Gilles, Just, Silaghi, Pradel, Passos, et al., 2008)
Portugal	*Ctenophthalmus sp*	Rodent	(De Sousa et al., 2006)
Australia, Democratic Republic of Congo	*Echidnophaga gallinacea*	Poultry, dog, cat	(Sackal et al., 2008; Schloderer et al., 2006)
Australia	*Spilopsyllus cuniculi*	Cat, rabbit	(Schloderer et al., 2006)
Brazil	*Polygenis atopus*	Dog, cat, opossum	(Horta et al., 2007)
Democratic Republic of Congo	*Tunga penetrans*	Dog, cat, pig	(Sackal et al., 2008)
Democratic Republic of Congo	*Xenopsylla brasiliensis*	Rodent	(Sackal et al., 2008)

Brazil	*Polygenis atopus*	Dog, cat, opossum	(Horta et al., 2007)
Algeria, Indonesia, USA	*Xenopsylla cheopis*	Rodent	(Bitam et al., 2009; Eremeeva et al., 2008; Jiang et al., 2006)
Japan	*Haemaphysalis flava*	Cat	(Ishikura et al., 2003)
Japan	*Haemaphysalis kitaokai*	Cattle, deer	(Ishikura et al., 2003)
Brazil, Chile	*Rhipicephalus sanguineus*	Dog, horse	(Abarca et al., 2013; Oliveira et al., 2008)
Japan	*Amblyomma cajennense*	Dog, horse	(Ishikura et al., 2003)
Taiwan	*Ixodes granulatus*	Shrew	(Tsui et al., 2007)
Japan	*Ixodes ovatus*	Cat	(Ishikura et al., 2003)
USA	*Carios capensis*	Seabird	(Reeves et al., 2006)
Croatia	*Haemaphysalis sulcata*	Sheep, goat	(Duh et al., 2006)
Taiwan	*Leptotrombidium deliense*	Rat	(Tsui et al., 2007)
Taiwan	*Mesostigmata*	Rat	(Tsui et al., 2007)
Australia	*Liposcelis bostrychophila*	-	(Behar et al., 2010)
Cuba	*Dermacentor nitens*	Horse	(Díaz-Sánchez et al., 2021)
Saint Kitts and Nevis	*Aedes aegypti*	-	(Valentine et al., 2021)
Gabon	*Aedes albopictus*	-	(Socolovschi, Pagés, et al., 2012)
Ivory Coast	*Anopheles gambiae*	-	(Socolovschi, Pages, et al., 2012)
China	*Anopheles sinensis*	-	(Zhang et al., 2014)
USA	*Anopheles punctipennis*	-	(Barua et al., 2020)
Saint Kitts and Nevis	*Culex quinquefasciatus*	-	(Valentine et al., 2021)

Table 2 Reported human cases with *R. felis* infection with clinical manifestations

Country	Reported year	Manifestation	Reference
USA	1994	Fever, headache, myalgia, rash, stiff neck	(Schriefer et al., 1994)
Mexico	2000	Fever, exanthema, headache, myalgia, abdominal pain, nuchal rigidity, fatigue, photophobia, arthralgia, vomiting, diarrhea	(Zavala-Velázquez et al., 2000)
France	2001	Fever, rash	(Raoult et al., 2001)
Brazil	2001	Fever, rash	(Raoult et al., 2001)
Germany	2002	Fever, fatigue, headache, cutaneous lesion, rash	(Richter et al., 2002)
Thailand	2003	Fever	(Parola et al., 2003)
South Korea	2005	Acute febrile illness	(Y.-J. Choi et al., 2005)
Spain	2006	Fever	(Nogueras et al., 2006)
Tunisia	2006	Fever, eschar, cutaneous rash	(Znazen et al., 2006)
Laos	2006	Fever	(Phongmany et al., 2006)
Egypt	2007	Fever	(Parker et al., 2007)
Taiwan	2008	Fever, chills, headache, fatigue, frequent micturition, numbness in hands, arthralgia	(K.-H. Tsai et al., 2008)
Sweden	2010	Fever, sore throat, vomiting, headache	(Lindblom et al., 2010)
Sri Lanka	2012	Fever, eschar, rash	(Angelakis et al., 2012)
Algeria	2012	Febrile exanthema	(Mokrani et al., 2012)
Morocco	2013	Fever	(Mediannikov et al., 2013)
Senegal	2013	Fever	(Mediannikov et al., 2013)
Mali	2013	Fever	(Mediannikov et al., 2013)
China	2014	Apparently healthy individuals	(Zhang et al., 2014)
Gabon	2015	Fever	(Mourembou et al., 2015)
Italy	2015	fever, retro-orbital headache, nausea, vomiting, and a mild maculopapular rash	(Sulis et al., 2015)
Indonesia	2020	Fever, headache,	(Mawuntu et al., 2020)
Slovakia	2021	Fever, tension headache, lymphadenitis, epistaxis, myalgia, and bone pains.	(Zubriková et al., 2021)

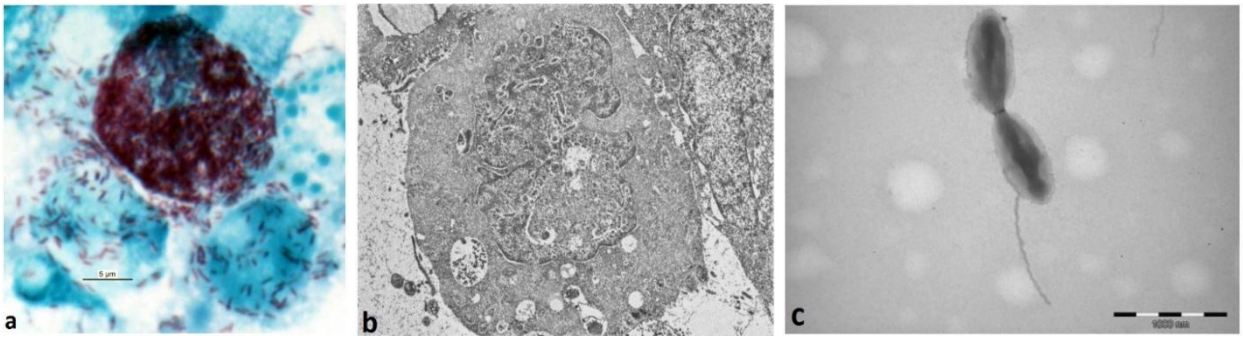

Figure 1. Giemenez staining and electron microscopy of *Rickettsia felis*. (a) *R. felis* growing in XTC2 cells. Giemenez staining magnification ×1000 (multiplication of *R. felis* within the nucleus. *R. felis* is pink or red with a blue-green background of the cytoplasm of the infected cell). Bar, 5 µm. **(b)** Electronic microscopy showing bacteria in the eukaryotic nucleus. **(c)** Identification of pili on *R. felis* by transmission electronic microscopy (negative staining). Image from Ogata H et al. New York Academy of Sciences 2005 (Ogata, Robert, et al., 2005).

Chapter 2. Trapping, morphological and molecular identification of mosquitoes

Introduction

Mosquitoes have been the most notorious arthropod vector of the world transmitting Zika, Dengue, Chikungunya, Japanese encephalitis, Yellow fever, Rift Valley fever, West Nile and Western Equine Encephalitis virus, along with several protozoans such as *Plasmodium*, nematodes, like *filaria*, and bacteria like *Rickettsia*. (Anoopkumar et al., 2019; Hernández-Triana et al., 2019). Hence, mosquitoes get the attention of the most widely distributed public health control and disease prevention programs throughout the world.

External morphological features constitute the conventional gold standard of identification of mosquito species. Unique anatomical landmarks are utilized in taxonomic keys to distinguish mosquito species (de Souza et al., 2020). However, morphological identification requires experienced taxonomists and the method itself is very tedious and time consuming. Moreover, mosquito is a delicate insect whose important anatomical features such as bristles, scales, and setae are damaged if handled improperly. As a result, there lies a risk of incomplete or misidentification of a species when a previously un-encountered species is found or when processing many specimens at the same time (Carter et al., 2019). In addition, morphological identification of species does not consider phenotypic plasticity and genetic variation of individuals. For example, members of the *Anopheles maculipennis* complex (Diptera: *Culicidae*), and possible hybrids, such as *Culex pipiens pipiens/Culex pipiens molestus* (Diptera: *Culicidae*), present a major problem in morphological identification (Werblow et al., 2016). Moreover, cryptic species complex appear very much indistinguishable morphologically though they differ in their capacity to transmit diseases (Walton et al., 1999). Only morphological classification under-represent the true diversity

of mosquito genera, whereas recent phylogenetic studies suggest presence of paraphyletic and polyphyletic taxa among these genera (Helmersson, 2013).

Most of the taxonomic keys are limited to adult female mosquitoes and fourth instar larvae because many of the morphological characteristics are not well developed in certain gender and in early larval stages (Hebert, Cywinska, et al., 2003). These limitations restrict the applicability of existing taxonomic keys for the identification of certain mosquito species. But the proper identification of mosquito species is essential in the epidemiological studies of mosquito-borne disease transmission. Proper knowledge of a species allows the investigation of other aspects of biology that differ between species such as larval ecology, resting behavior and insecticide resistance, that are vital to the implementation of effective vector control strategy (Walton et al., 1999). This necessity and the contemporary improvement in the field of genetics opened the horizon of molecular identification of mosquitoes. Using a short-standardized sequence of DNA as a genetic marker for species identification started the era of mosquito DNA Barcoding. Genetic analyses not only accurately separated species, determined synonymies, helped to describe new taxa, clarified subspecies status and resolved cryptic species groups, but also detected damaged specimens, and life stages with less morphological features (Cane et al., 2020). Moreover, DNA data are interoperable with previous DNA records which links to rich metadata on location and date of isolation. Information about population structure and movement of vector species would ultimately improve the understanding of the spatial epidemiology of mosquito-borne diseases (Carter et al., 2019).

Different molecular genetic markers has been used as the barcoding region such as, the nuclear internal transcribed spacer 2 (ITS2), cytochrome b oxidase, whole mitochondrial genomes, mitochondrial cytochrome c oxidase subunit 1 (COI), 12S rRNA, nicotinamide adenine

dinucleotide dehydrogenase subunit 4 (ND4), ND6, Dominant receptor (D2), elongation factor-1 alpha (EF-1a), acetylcholinesterase 2 (ace-2), alpha amylase, and zinc finger (Batovska et al., 2016; Ruiz-Lopez et al., 2012). Among these targets COI gene is most popular due to its easy applicability as a universal set of primers and its ability to provide a higher sequence variation (Chan et al., 2014). The Barcode of Life Database (BOLD) has selected this COI region as the "Universal" or "*Folmer*" region as the standard marker for DNA barcoding (Batovska et al., 2016).

Earlier, Hebert et al. in 2003 showed by analysis of cytochrome c oxidase subunit I (COI) sequences that DNA barcoding can distinguish some species among closely related species across diverse phyla in the animal kingdom (Hebert, Ratnasingham, et al., 2003). In addition, the mitochondrial gene encoding COI can determine phylogenetic relationships between closely related families, genera, and species of insects and can differentiate between subspecies (Palenko et al., 2004). So, we targeted this polymorphic mitochondrial DNA (mtDNA) gene to develop a new set of universal primers specific for mosquito genera. The existing universal primer system gives artefact results if whole mosquito is used as the specimen for DNA extraction. In this study, we sought to improve current universal primer system for proper molecular diagnosis of mosquito species by targeting the COI-DNA region which could be utilized in mosquito surveillance program.

We demonstrated that the COI sequences derived by applying our primer system enabled the reliable assignment of mosquitoes to more proper taxonomic categories. In medical entomology, molecular diagnosis of species by DNA barcoding is crucial in the identification of all life stages, from eggs to adults. This tool will deliver reliable identification, aid in mosquito surveillance, and reduce interception response times, all of which may be vital to the successful mosquito control program.

Methods and Materials

Mosquito trapping

During 2019 and 2020, adult live mosquitoes were trapped across Auburn, Alabama as part of the *Rickettsia felis* Surveillance Program (Barua et al., 2020; Hoque et al., 2020). 7 locations were selected to trap mosquitoes: hospital premises, animal farm, construction site, public parks, housing estate, and academic building area to maximize the diversity of species of the trapped mosquitoes. The geographical sites where the mosquitoes were trapped are shown in **Figure 2**.

Mosquitoes from the construction site, public parks, housing estate, and academic buildings would represent the mosquito-borne human pathogens whereas mosquitoes trapped from the veterinary clinic neighborhood, the animal farm would represent the mosquito-borne pathogens of veterinary importance. At the same time, mosquito trapping from these sites represented the demography of mosquitoes at Auburn. All the mosquito collection sites were humid, ambient in temperature, and were near water bodies.

Mosquitoes were trapped in two phases: during the month of October 2019 and from June to September of 2020. Four CO2-baited BG-2-Sentinel Mosquito Trap (BioQuip, Rancho Dominguez, CA, USA) and six UV light baited New Standard Miniature Light Trap (John W. Hock, Florida, USA) (**Figure 3**) were used to trap mosquitoes. Mosquito trappers were operated from 18.00 hours to 8.00 hours of the next day to catch both the daytime and night biters.

The mosquito trappers were powered by rechargeable DC batteries. Dry ice in cool boxes as a source of CO_2 and BG Lure (From BioQuip, Rancho Dominguez, CA, USA) were used as mosquito attractants with baited BG-2-Sentinel Mosquito Trap. A closed cool box with 2 small holes on the lid, equipped with 400-500g of dry ice was used with each BG-2-Sentinel Mosquito Trap. The cool box was placed beside the trap so that the airflow created by the running fan was

sucked into the mosquito collection net. Mosquitoes were trapped along with the air inside the collection net. BG Lure was placed inside the BG traps, on the wall of the trap. UV light was used with New Standard Miniature Light Trap to attract mosquitoes.

Mosquitoes were trapped on 4 to 7 consecutive nights to collect a valid index of the mosquito population of an area. Mosquito trappers were placed 3-4 feet above the ground, near marshy land away from strong wind. The trapping location was humid and dark. Trapping was not operated when it was rainy or was too cold (below 10^0 C). The average temperature was between 16.5^0 C and 30^0 C, humidity 50% - 95%, and rainfall 2.17”-3.35” during the mosquito trapping season.

A total of 560 mosquitoes were trapped in 2019 and another 2064 mosquitoes were trapped during 2020 from Auburn, Alabama. Following trapping, these mosquitoes were transported alive in cool boxes to the laboratory.

Morphological identification, sorting and pooling of mosquitoes

After trapping, live mosquitoes were taken to the laboratory, they were processed immediately or stored at -20^0 C and processed later. For immediate processing, mosquitoes were kept at 4^0 C for 30 minutes to immobilize them. A chill table was devised conveniently by putting ice underneath a petri dish. Then these mosquitoes were taken on this chill table and were observed under a stereomicroscope to identify them according to their anatomical features. An AmScope 3.5X-180X Simul-Focal Stereo Zoom Microscope (**Figure 4**) was used to detect the mosquito species. Standard taxonomic keys were followed to sort out mosquito species morphologically (Burkett-Cadena, 2013; Willott & Ramberg, 2007).

Mosquitoes that were stored to process later, were kept at -20^0 C in petri dishes making them airtight with parafilm. Individual petri dish was used for each day and for each location. Petri

dishes were labeled properly according to trapping time and trapping location. These mosquitoes were taken out, sorted into species observing with the stereomicroscope on a chill table following the same protocol mentioned above.

After morphological identification, 8-15 mosquitoes of the same species were taken in a microcentrifuge tube. 57 mosquito pools were made out of the mosquitoes trapped in 2019 and another 218 pools were made out of the mosquitoes trapped in 2020. These pools of mosquitoes were used for the detection of mosquito-borne pathogens.

At the same time, a single mosquito representing each mosquito species was taken into an individual microcentrifuge tube for molecular detection of the species and for the validation of the anatomical identification. A total of 51 single mosquito samples were made out of the total trapped mosquitoes. Along with these single mosquito samples, another 77 mosquito samples were shared with our laboratory from Saint Kitts and Nevis, West Indies as a part of the *Rickettsia felis* Surveillance Program among mosquitoes in this island (Valentine et al., 2021). These mosquito samples were tested for their molecular identification too.

Washing and homogenization of the mosquitoes

Following sorting the mosquitoes into microcentrifuge tubes, they were undergone further processing. The mosquito washing procedure was performed according to the protocol mentioned in (Barua et al., 2020). In short, the mosquitoes were washed once with 1X PBS, followed by incubation at room temperature with 1 mL of 70% ethanol for 10 minutes. Then the 70% ethanol was discarded and the mosquitoes were washed with 1 mL 1X PBS 4 times to wash away the surface contaminants. 800 µL 1X PBS and three zirconia beads were added to the tube. Finally, the sample was homogenized with Precellys 24 lysis and homogenization at 5000 rpm for 15 seconds (**Figure 4**). Following homogenization, the sample was divided into 2 tubes, each

microcentrifuge tube containing 400 µL homogenate. Then these homogenates were stored at -20^0 C until DNA extraction.

While processing single mosquito samples, the same protocol was followed. However, the single mosquito was homogenized in 400 µL of 1X PBS and it was stored at -20^0 C until DNA extraction.

DNA extraction

The High-Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA) was used to extract total nucleic acids from whole mosquito homogenate according to the manufacturer's instructions and following a similar protocol described before (Hoque et al., 2021; Poudel et al., 2020a). In brief, mosquito homogenate (400 µL) was mixed with an equal volume of binding buffer (400 µL) followed by digestion with 80 µL proteinase K (10% of total volume). Cell lysis was carried out by incubation at 72^0 C for 20 minutes with shaking at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). Afterward, 200 µL of Isopropanol (Avantor Performance Materials, Center Valley, PA) was added to the mixture and vortexed thoroughly. Then the kit filter was inserted into a 2 mL collection tube and the aqueous solution was transferred into the filtered collection tube followed by centrifugation at 8,000 rpm for 1 minute. The flow-through was discarded and 500 µL of inhibitor removal buffer was added followed by centrifugation at 8,000 rpm for 1 minute. The flow-through was discarded and the filter was washed with 1,000 µL of washing buffer in two steps. In the first washing step, 600 µL washing buffer was added to the filter column followed by centrifugation at 8,000 rpm for 1 minute and was discarded. In the second step, 400 µL washing buffer was added followed by centrifugation at 13,000 rpm for 2 minutes and was discarded. Then the filter column was inserted into a new 2 mL collection tube. The genomic DNA was eluted with 200 µL elution buffer pre-warmed at 72^0

C in 2 steps. At first, 100 µL elution buffer was added directly to the middle of the filter, incubated at 72^0 C for 5 minutes, and centrifuged at 8,000 rpm for 1 minute. Then another 100 µL elution buffer was added and centrifuged in the same manner. The extracted nucleic acid was used for the molecular detection of mosquito species and mosquito-borne pathogens.

Primer design for molecular detection of mosquito species

Following morphological identification, mosquitoes were identified with the molecular method to validate the anatomical identification. The mitochondrial Cytochrome Oxidase C Subunit 1 (COI) was taken as the target region of DNA to barcode the mosquitoes. The COI region was targeted as the DNA Barcoding region because (1) it can distinguish species among closely related species across diverse phyla in the animal kingdom, (2) it can determine phylogenetic relationships between closely related families, genera, and species of insects like mosquitoes, (3) it can differentiate between subspecies, and (4) it can separate and detect the species within a cryptic species complex which is very much challenging to identify by looking at the morphological features.

For DNA barcoding of the mosquitoes, at first, the LCO 1490 and HCO 2198 primers were used as described in *Folmer et al. (1994)*. These primers amplified a region of 709 base pairs (including primers) of the mitochondrial COI of diverse metazoa. The numbers (1490 and 2198) refer to the position of the base pairs in the *D. yakuba* 5' nucleotide (Folmer et al., 1994). But it failed to yield consistent positive amplicons. So, 2 new sets of primers were designed targeting the universal *Folmer* region for DNA barcoding of mosquito species.

To design new primers, complete DNA sequences of mitochondrial Cytochrome Oxidase C Subunit 1 for widely available 38 species from 11 Genus were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). The genus, species, and GenBank accession numbers

are listed in **Table 3**. These mosquito mtDNA sequences were the longest available COI sequences during the primer design work. Then these sequences were aligned with VectorNTI using ClastalW to find out the most conserved region (**Figure 5**). After alignment, 7 sequences fell in a few base pairs short. So, finally, 31 sequences were kept in the alignment to design the primers.

DNA sequencing with the existing *Folmer* primer system sometimes yielded in artifact sequences of moths, midgets, drosophila, and other non-mosquito organisms. To increase the specificity of our primer system, some non-mosquito COI sequences were included in the alignment. Polymorphic region closely aligning these non-mosquito sequences were avoided during designing the primers. A 712 bp fragment in the 5' end of the mitochondrial Cytochrome Oxidase C Subunit 1 (COI) barcode region was selected as the target region for the In-House primer (AU-COI). This fragment of DNA contains highly polymorphic region in between two conserved areas. The design was based upon the existing *Folmer et al. 1994* primers (LCO1490 and HCO 2198) and we modified the forward and reverse primer sequences to design our AU-COI primers.

In the case of the In-House Forward Primer (AU-COI-F), a total of 3 degenerate bases were inserted in the primer. 1 base was replaced with a degenerate base in 2 codons. The base pair was replaced at the 2nd position of each codon. At position 8 of the alignment, A was replaced with W and at position 20, A was replaced with R. Furthermore, the primer length was increased bi-directionally by 3 base pairs. As a result, the *Folmer* Forward Primer (LCO 1490) was modified from a length of 25 bases to a 31 base long In-House Forward Primer (AU-COI-F). At position 29 (3' end of the forward primer) another degenerate base W was added. For the In-House Reverse Primer (AU-COI-R), 6 degenerate bases at the wobble positions of the codons were inserted. At positions 692 and 695 T was replaced with Y, at position 698, 704, and 710 T was replaced with

W, and at position 701 C was replaced with Y. As a result, the *Folmer* Reverse Primer (HCO 2198) was modified into a 26 base long In-House Reverse Primer (AU-COI-R). The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer sequences of both *Folmer* and In-House primer (AU-COI) systems are shown in **Table 4**.

Molecular identification of mosquitoes and DNA sequencing

Following DNA extraction and design of the primer, PCR was run with the single mosquito DNAs. Both the *Folmer* (LCO1490 and HCO 2198) and In-House (AU-COI-F and AU-COI-R) (**Table 4**) primer systems were used for PCR. Mosquito DNA samples were used to run SYBR Green PCR on a LightCycler 480-II Thermocycler (Roche Diagnostics GmbH, Mannheim, Germany) using a high-stringency 18-cycle step-down temperature protocol as described in previous studies (Hoque et al., 2021; Poudel et al., 2020a). In brief, for each PCR reaction, 10 µL of the extracted DNA was added to a 10 µL reaction mixture containing 5X PCR SYBR buffer, 400 µM dNTP (Roche Diagnostics GmbH), 0.34 units of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1 µM of each forward and reverse primer (Integrated DNA Technologies, Coralville, IA, USA), and a final volume of molecular grade nuclease-free water.

PCR amplified a 712 bp fragment in the 5' region of the COI of the mitochondrial genome with AU-COI primer (In-House primer) and a 709 bp fragment of the COI region in case of the *Folmer* primer system (Folmer et al., 1994). PCR products for the samples were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for Sanger sequencing using both ends of the primers, followed by BLASTn to identify the mosquito species.

Assessment of the sensitivity of the designed PCR system in this study

Following SYBR-qPCR amplification, random PCR products were purified with QIAquick PCR Purification Kit according to manufacturer's protocol. In brief, PCR products were taken into microcentrifuge tubes and 5 volumes of Buffer PB was added to 1 volume of the PCR product. Microfuge tubes were centrifuged at 2,000 rpm for 1 minute, vortexed and again centrifuged at 2,000 rpm for 1 minute. The QIAquick spin column was inserted into a 2 mL collection tube and the aqueous solution was transferred into the column followed by centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and 750 µL Buffer PE was added to the column followed by centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and the column was taken into a new set of 2 mL microfuge tube. The DNA was eluted with Buffer EB. 50 µL of Elution Buffer was added at the center of the QIAquick membrane and the column was centrifuged for 1 min at 13,000 rpm. The purified DNA was used for PicoGreen Assay and assessment of the sensitivity at different copy numbers of the DNA.

After the purification, concentration of the DNA was measured with Quant-iT™ PicoGreen ® dsDNA assay Kits according to manufacturer's protocol. In brief, a standard curve was generated with different concentrations of Lambda DNA standard in Quant-iT™ PicoGreen dsDNA reagent and 1X TE buffer with a SpectraMax® iD3 Multi-Mode Microplate Reader. From the standard curve and fluorescence intensity, the concentration of the undiluted purified DNAs was measured. Standards were made with different copy numbers for each DNA. The copy numbers were 10^4, 10^3, 10^2, 10^1, and 10^0 copies.

Results

The AU-COI PCR system was validated to show sensitivity of a single copy of DNA/10 µL reaction (*Ae. japonicas, An. quadrimaculatus, Cx. nigripalpus, Cx. usquatissimus, Or. alba, Ps. ferox, and Ur. sapphirina*), and a sensitivity of 10 copies (*An. punctipennis).*

In, total, 2,624 mosquitoes were trapped in this study for validation of COI-based PCR and *R. felis* detection (Chapter 3). In total, 128 representative mosquito DNAs (51 from Auburn and 77 from Saint Kitts and Nevis) were used to test the *Folmer et al. 1994* and In-House (AU-COI) primer systems.

Eight mosquito species were successfully identified by morphology and two PCR systems. In addition, seven mosquito species were correctly identified by two PCR systems, but not by the morphology. Furthermore, 9 mosquito species were only accurately identified by our in-house (AU-COI) PCR, not by the *Folmer et al. 1994* one and the morphology (**Table 5**).

For those mosquito species successfully determined by both PCR systems, the in-house PCR system constantly showed a high copy number (lower ct values) than the *Folmer* one (**Figure 6**). In a similar trend, the DNA sequencing data had a higher quality in AU-COI PCR than the *Folmer*'s primers (1994).

Discussion

Among different methods of identification of mosquito species (i.e. dichotomous key, alloenzymes, and DNA-based methods) genetic techniques are relatively free from the subjectivity of identification of morphological features and can detect cryptic species complexes that are often overlooked (Batovska et al., 2016). Among different DNA-based target regions, mitochondrial DNA is popular to identify mosquitoes. This target has a precise variability in the conserved region which detects mosquitoes at their species level (Walton et al., 1999). Additionally, mitochondrial

genes are often the sequences of choice for phylogenetic studies as they are (1) highly conserved among phyla, (2) maternally inherited, (3) present in high copy number, and (4) because mtDNA evolves faster than nuclear DNA (Morlais & Severson, 2002). Moreover, mitochondrial genes are shared across diverse taxa and do not contain indels (Stoeckle, 2003). In addition, mitochondrial DNA (mtDNA) functions as a molecular clock where transversions accumulate in a linear fashion over time (Wang et al., 2012a).

Cytochrome Oxidase C Subunit 1 (COI) is the largest of the mitochondrial-encoded cytochrome oxidase subunits and its protein sequence contains highly conserved functional domains and variable regions (Morlais & Severson, 2002). It generally lacks indels, and its third position nucleotides show a high incidence of base substitutions. Changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene, aiding the resolution of deeper taxonomic affinities and primer design (Cywinska et al., 2006). Moreover, in comparison to other mitochondrial genes, the phylogenetic signal from COI has greater output. It can separate species phylogeography and discriminate closely related species. Therefore, universal primers developed for this gene are very robust and this marker often is used for evolutionary study, as well as for a bio-identification system (Helmersson, 2013).

In 2019, Hernández-Triana LM et al. showed that COI DNA barcoding with the standard *Folmer* primers had identification problems within the genus *Culiseta* (*Cs. fumipennis, Cs. Litorea,* and *Cs. morsitans*) (Hernández-Triana et al., 2019). Similarly, the primers provided in *Folmer et al. 1994* failed to yield consistent positive amplicons in our study. Earlier, Wong et al. (2014) warned that the abdominal tissue of mosquitoes should be avoided as too many inhibitors from the intestines may be present (Wong et al., 2014). Hence, we tried to improve the existing primer system making it more specific for the mosquito species. We aimed to design a primer that would

be able to detect mosquito species even when the whole mosquito sample would be used to extract DNA. Moreover, it would be useful when specimens are found in a poor state of preservation which is an essential step for the establishment of control measures (Hernández-Triana et al., 2019). It is also important to identify mosquitoes clearly to generate information on their distribution and the potential risk for the transmission of disease (Werblow et al., 2016).

DNA sequence analysis of a uniform target gene to enable species identification is analogous to the Uniform Product Code on manufactured goods, hence termed as *DNA barcoding.* DNA-based identification is important in flagging specimens that represent undescribed taxa (Stoeckle, 2003). This technology is unaffected by morphological variation between different stages of the life cycle and it allows the homogenization or calibration of the taxonomic units identified in different areas. DNA barcode technology generally precisely identifies mosquitoes which is vital to the accuracy of a surveillance program (Batovska et al., 2016), thereby it greatly reduces the burden on the taxonomists (Wang et al., 2012b).

The gold standard for any taxonomic system is its ability to deliver accurate species identifications. Parameters that may influence the amplification are the primer pairs, the amount of tissue used, and the polymerase used (Werblow et al., 2016). First, the standard barcoding primers by *Folmer et al. 1994* were used in our study. However, the amplification results showed great variation, inconsistent quality of DNA sequences (**Figure 6**), and a blast search revealed that the primers, because of their low specificity, are unsuited for DNA barcoding of varieties of mosquitoes using qPCR. As this did not serve our purpose of mosquito species identification, a new and effective primer system was needed to conduct our surveillance.

Our devised AU-COI primer system consistently showed a more robust, sensitive, and specific diagnosis of the mosquito species. AU-COI primer system detected around 20% more

species than the *Folmer et al. 1994* primers was able to detect from the very same cohort of mosquito samples. AU-COI primer system showed more sensitivity than the *Folmer et al. 1994* primer in detecting very low concentration of DNA, even 1 copy/10 µL DNA was detected with the AU-COI primer system. Simultaneously, the AU-COI system persistently produced high quality DNA sequences in case of most of the mosquito samples. Blast searches revealed even previously unreported species of mosquitoes in the sites of the study.

Past phylogenetic work has often focused on mitochondrial genes encoding ribosomal (12S, 16S) DNA, but their use in broad taxonomic analyses is constrained by the prevalence of insertions and deletions (indels) which greatly complicate sequence alignments. The 13 protein-coding genes in the animal mitochondrial genome are better targets because indels are rare since most lead to a shift in the reading frame. Moreover, the mitochondrial cytochrome c oxidase I gene (COI) has two important advantages. First, the universal primers for this gene are very robust, enabling recovery of its 5' end from representatives of most animal phyla, let alone mosquito genera. Second, COI appears to possess a greater range of phylogenetic signals than any other mitochondrial gene. In addition, its third-position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA. In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species but also phylogeographic groups within a single species (Hebert, Cywinska, et al., 2003).

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Table 3 Mosquito species considered in designing COI PCR to barcode mosquitoes

Genus	Species	GenBank Accession Number
Aedes	*Ae. aegypti*	MK575474.1
	Ae. albopictus	MN513368.1
	Ae. busckii	MN626443.1
	Ae. geniculatus	MT993491.1
	Ae. japonicas	KF211505.1
	Ae. vexans	MT993484.1
Anopheles	*An. albimanus*	KC354824.1
	An. balabacensis	MH032639.1
	An. crucians	MT757853.1
	An. funestus	MT917175.1
	An. gambiae	MG753769.1
	An. pseudopunctipennis	KC354820.1
	An. quadrimaculatus	L04272.1
	An. stephensi	KT899888.1
Culex	*Cx. erraticus*	MH129001.1
	Cx. nigripalpus	NC_037823.1
	Cx. pipiens molestus	MN389459.1
	Cx. quinquefasciatus	MK575480.1
	Cx. sitiens	NC_054318.1
	Cx tarsalis	AF425847.1
	Cx. tritaeniorhynchus	KT852976.1
	Cx. univittatus	LC102144.1
	Cx. vishnui	MH374857.1
Culiseta	*Cs. annulata*	MT993485.1
	Cs. incidens	KP293420.1
	Cs. melanura	JX259943.1
Coquillettidia	*Cq. perturbans*	GU013591.1
Mansonia	*Ma. annulata*	HQ341635.1
	Ma. uniformis	NC_047479.1
Ochlerotatus	*Oc. detritus*	MT993478.1
	Oc. fluvus	MK575476.1
	Oc. taeniorhynchus	MN626442.1
Psorophora	*Ps. cingulata*	KM592989.1
	Ps. ferox	MK575485.1
Toxorhynchites	*Tx. theobaldi*	KY782650.1
	Tx. rutilus	AF425849.1
Uranotaenia	*Ur. unguiculata*	MT993499.1
Wyeomyia	*Wy. confusa*	NC_044663.1

Table 4 Primers used in this study targeting the mitochondrial COI

Primer	**Sequences (5' to 3')**	**Reference**
LCO 1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	(*Folmer et al.*, 1994)
HCO 2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	
AU-COI-F	5'-TATTTTCWACAAATCATAARGATATTGGWAC-3'	Designed in this study
AU-COI-R	5'-TAWACTTCWGGRTGWCCRAARAATCA-3'	

Table 5 Mosquito species identified by morphology and molecular identification system

Mosquito	Morphology	*Folmer et al 1994* primer (ct)	AU-COI primer (ct)	Successful Identification
SK-AB	*Ae. busckii*	*Ae. busckii* (18.0)	*Ae. busckii* (10.5)	Three systems
AU-S203	*An. crucians*	*An. crucians* (9.6)	*An. crucians* (25.0)	
AU-S137	*An. punctipennis*	*An. punctipennis* (21.7)	*An. punctipennis* (10.0)	
SK-D1	*Dei. magnus*	*Dei. magnus* (23.6)	*Dei. magnus* (7.9)	
AU-S192	*Ps. ferox*	*Ps. ferox* (17.9)	*Ps. ferox* (10.1)	
AU-S199	*Ps. howardii*	*Ps. howardii* (15.7)	*Ps. howardii* (2.0)	
AU-S182	*Ur. sapphirina*	*Ur. sapphirina* (6.4)	*Ur. sapphirina* (5.0)	
AU-S115	*Cx. pipiens*	*Cx. pipiens* (25.0)	*Cx. pipiens* (17.0)	
SK-AT2	*Ae. tortilis*	*Oc. tortilis* (17.5)	*Oc. tortilis* (6.0)	Two COI systems; Morphology only at genus level or at wrong species
SK-AA1	*An. albimanus*	*Ae. aegypti* (19.0)	*Ae. aegypti* (4.0)	
SK-C2	*Cx. nigripalpus*	*Cx. pipiens* (25.0)	*Cx. pipiens* (9.0)	
AU-S201	*Cx. nigripalpus*	*Cx. erraticus* (23.0)	*Cx. erraticus* (25.0)	
AU-S202	*Cx. nigripalpus*	*Cx. erraticus* (25.0)	*Cx. erraticus* (24.2)	
AU-S156	*Cx.* spp.	*Cx. pipiens* (16.4)	*Cx. pipiens* (25.0)	
AU-S169	*Cs. melanura*	*Cx. erraticus* (23.0)	*Cx. erraticus* (25.0)	
AU-S183	NI	*Cx. nigripalpus* (15.8)	*Cx. nigripalpus* (5.0)	
AU-S186	*Or.* spp.	*Or. alba* (5.0)	*Or. alba* (5.0)	
AU-S196	*Or. signifera*	*Or. alba* (4.0)	*Or. alba* (3.0)	
AU-S149	*Ps.* spp.	*Ps. confinnis* (18.7)	*Ps. confinnis* (5.0)	
AU-S181	*Cx. tarsalis*	*Cx. coronator* (22.7)	*Cx. coronator* (5.0)	
AU-S184	*Cx.* spp.	NI	*Cx. erraticus* (6.0)	Our COI, not old COI; Morphology only at genus level or at wrong species
AU-S198	*An. quadrimaculatus*	NI	*An. quadrimaculatus* (6.0)	
AU-S166	*Ae. japonicus*	NI	*Ae. japonicas* (10.8)	
SK-14	*Ae.* spp.	NI	*Ae. aegypti* (20.7)	
SK-1	*Ae. taeniorhynchus*	NI	*Oc. taeniorhynchus* (13.4)	
AU-S172	*Cx. coronator*	NI	*Cx. usquatissimus* (24.4)	
AU-S187	*Cx. coronator*	NI	*Cx. usquatus* (9.4)	
AU-S123	*Cx. pipiens*	NI	*Cx. pipiens* (20.3)	
AU-S200	*Cx.* spp.	NI	*Cx. pipiens* (5.0)	
SK-28	*Ps. pygmaea*	NI	*Ps. pygmaea* (14.9)	
SK-P1	*Ps. pygmaea*	NI	*Ps. cingulate* (8.0)	
AU-S197	NI	NI	*Oc. triseriatus* (8.2)	
SK-T	*Tx. guadeloupensis*	NI	*Tx. theobaldi* (16.6)	

Figure 2 Sites of mosquito collection at Auburn, Alabama. (1) Auburn University College of Veterinary Medicine Campus, (2) Donald E. Davis Arboratum, (3) Peet Theatre, (4) Chewacla Drive, (5) Town Creek Park, (6) Paces at the Estates, (7) Felton Little Park.

Figure 3 Mosquito trappers used to trap mosquitoes at Auburn in Alabama. (Left panel): CO2-baited BG-2-Sentinel Mosquito Trap. (Right panel): UV light baited New Standard Miniature Mosquito Trap.

Figure 4 Stereomicroscope and Homogenizer used for morphological identification and homogenization of mosquitoes. (Left panel): Stereo Zoom Microscope for morphological identification and dissection of mosquitoes. (Right Panel): Precellys 24 Homogenizer.

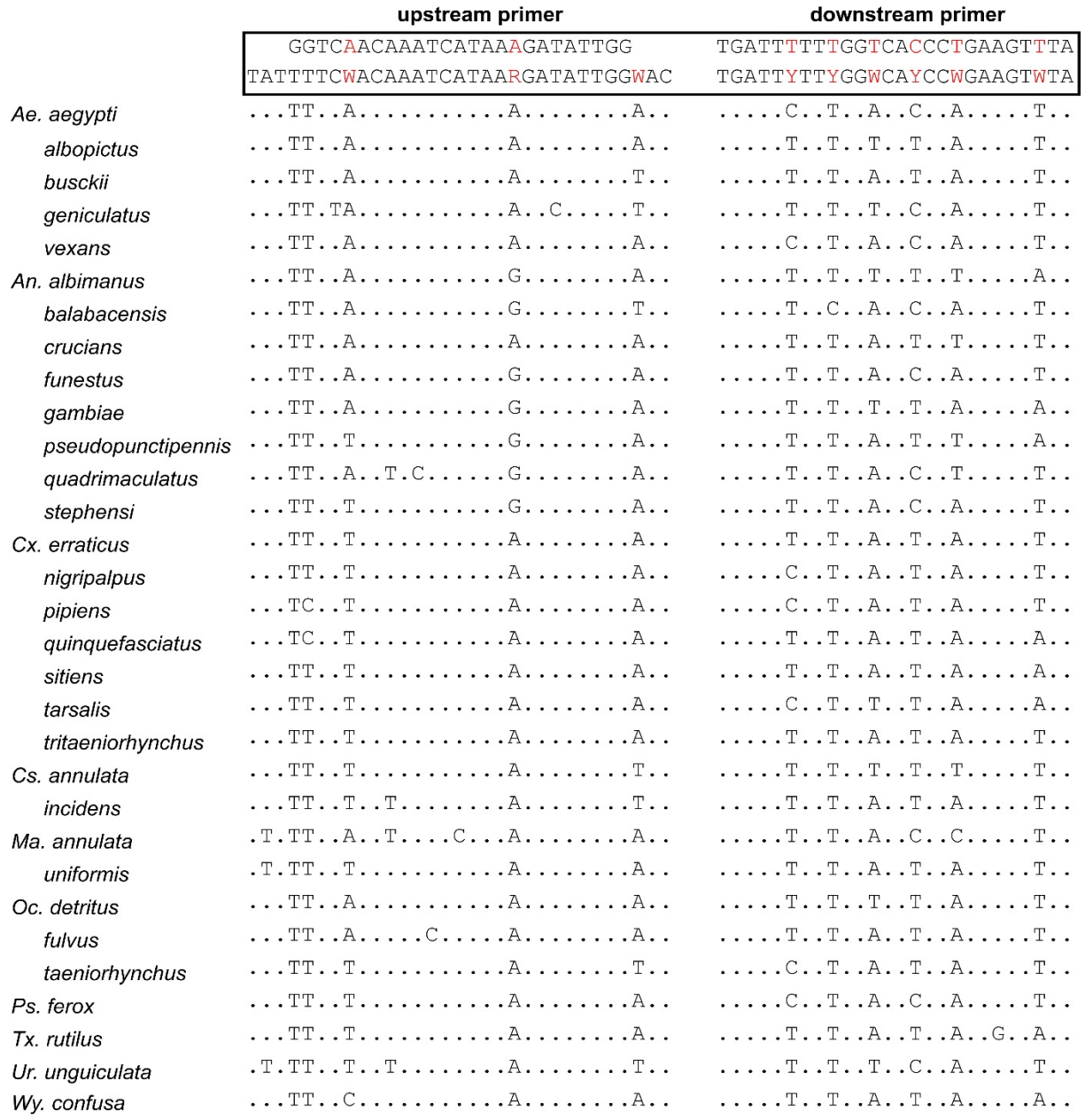

Figure 5 Alignment of COI amplification targets of medically important mosquito species with primers. The primers are shown as uppermost boxed sequences, and the first row are the primer sequences from *Folmer et al.* and the second row is from this study. The upstream primers are used as shown while the downstream primers are used as antisense oligonucleotides. Dots indicate nucleotides identical to those of primers. Nine degenerate sequences are used in the primers from this study. The amplicon regions between the primers are highly polymorphic among different mosquito species (data not shown). W represents A or T, R represents A or G, and Y represents C or T.

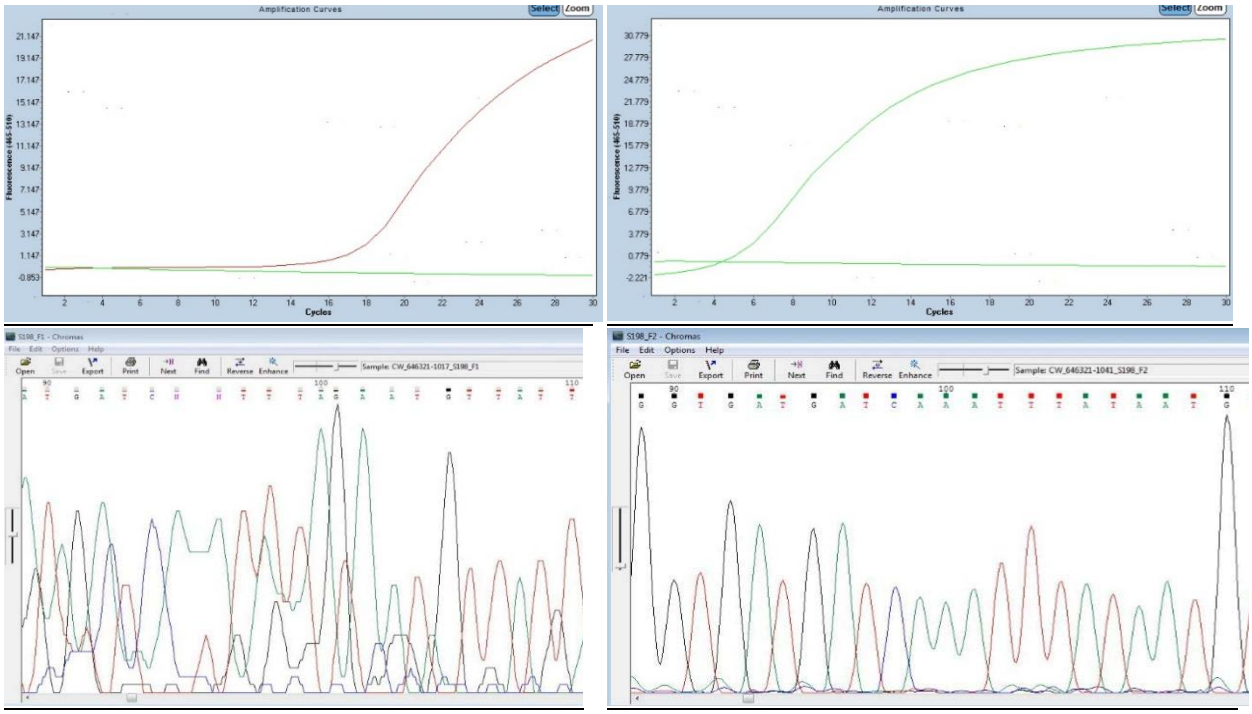

Figure 6 Amplification curve and Sanger sequence of SYBR PCR of S198 mosquito (*An. quadrimaculatus*) DNA. The ct value with the *Folmer et al.* Primer (LCO 1490 and HCO 2198) is on top left panel and the ct value for In-House Primer (AU-COI) system is on top right panel. ct value for *Folmer et al.* Primer is 18, whereas, the ct value for the AU-COI is 5. Sanger sequence of the corresponding PCR products are shown in the bottom panel. AU-COI system produces much clean result.

Chapter 3. First report of *Rickettsia felis* in mosquitoes, USA

Introduction

The recently described *Rickettsia felis* is an emerging human pathogen causing flea-borne spotted fever (Angelakis et al., 2016). Although found in a wide range of arthropods including fleas, ticks, mites, and lice, the cat flea (*Ctenocephalides felis*) is currently believed to be the most likely primary vector of *R. felis*. There is growing evidence, however, that mosquitoes might be involved in *R. felis* transmission with the organism having been identified in a wide variety of mosquitoes in Africa and China (Mediannikov et al., 2018); mouse-model experiments have indicated transmission of *R. felis* by *Anopheles gambiae* (Zhang et al., 2019); there is an association between malaria and flea-borne spotted fever cases in Africa (Mediannikov et al., 2018). Although *R. felis* has been demonstrated in a variety of mammals and arthropods in the USA, there is only one study on its presence in mosquitoes. The organism was not identified by PCR in pools of *Culex quinquefasciatus, Aedes albopictus, Culex pipiens* complex, *Anopheles punctipennis* and *Anopheles crucians* from Georgia (Anderson et al., 2019).

Methods and Materials

Mosquito trapping

Adult live mosquitoes were trapped throughout October 2019 on the campus of Auburn University College of Veterinary Medicine, Alabama with 6 UV light baited New Standard Miniature Light Trap (John W. Hock, Florida, USA) (**Figure 3**). Mosquito trappers were operated from 18.00 hours to 8.00 hours of the next day to catch both the daytime and night biters. A total

of 560 unfed adult mosquitoes were trapped. Following trapping, these mosquitoes were transported alive in cool boxes to the laboratory.

Morphological identification of mosquitoes

After live mosquitoes were taken to the laboratory, they were processed immediately following the above-mentioned protocol. In brief, they were paralyzed by keeping at 4^0 C for 30 minutes and then identified at their species level with a stereomicroscope. Mosquitoes were pooled (4 to14 per pool) according to species, sex, trap number, and collection site following standard taxonomic keys (Burkett-Cadena, 2013; Willott & Ramberg, 2007). A total of 57 mosquito pools were made out of these 560 mosquitoes. These pools of mosquitoes were used for the detection of *R. felis* in mosquitoes.

Washing, homogenization and DNA Extraction

Mosquito pools were washed and homogenized following the above-mentioned protocol. In brief, they were washed in 1X PBS for one minute; once in 70% ethanol for ten minutes; four washes in 1X PBS one minute each, respectively. DNA was extracted with High-Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA) following above-mentioned protocol. In brief, mosquito homogenate (400 µL) was mixed with an equal volume of binding buffer followed by digestion with proteinase K (10% of total volume). Nucleic acid was eluted in the final volume of 100 µL elution buffer.

Detection of *R. felis* DNA by PCRs

Three previously validated quantitative PCR assays, mainly a *gltA*-based *Rickettsia* FRET-PCR (Zhang et al., 2019) for citrate synthase, a nested PCR targeting the *gltA* of *Rickettsia* (Zhang

et al., 2019), and a *R. felis* species-specific *BioB*-based PCR (Anderson et al., 2019) for biotin synthase genes were used to detect *Rickettsia* DNA in the samples. A list of the primers are enlisted in **Table 6**.

Ten µL of each DNA sample was screened initially with the highly sensitive *Rickettsia* FRET qPCR. 10 µL of the positive DNA samples were further used for nested PCR and another 10 µL for *R. felis* species-specific *BioB*-based PCRs. The *R. felis* DNA from a previous study (Zhang et al., 2019) and nuclease free water were used as positive and negative controls, respectively. The arbitrary five-fold dilution was also performed on the positive controls.

All PCR reactions were performed on a Roche Light Cycler 480 II thermocycler (Roche Diagnostics GmbH, Mannheim, Germany) as described in *Barua S et al. 2020* (Barua et al., 2020). In brief, 10 µL of the extracted DNA was added to a 10-µL reaction mixture containing 5x PCR FRET buffer, 400 µM dNTP (Roche Diagnostics GmbH), 0.34 units of Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1 µM of each forward and reverse primer (Integrated DNA Technologies, Coralville, IA, USA), and a final volume of molecular grade nuclease-free water.

The products of *Rickettsia*-positive PCRs were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for Sanger DNA sequencing using upstream and downstream primers. The nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) to obtain GenBank Accession numbers, and a phylogenetic tree was generated to compare the nucleotide sequences of *Rickettsia* identified in this study with those of *R. felis*-like organisms (Odhiambo et al., 2014) and other *Rickettsia* species (**Figure 7**). The mosquito species were included in the **Figure 7** to indicate which mosquito species harbor *R. felis* in this study. The

nucleotide sequences of the *gltA* PCR products were concatenated and aligned using CLUSTALW, and the phylogenetic inferences were obtained from a maximum likelihood analysis.

Results

After running the *R. felis* PCR system with 57 pooled mosquito DNAs, five mosquito pools were found positive for *R. felis* with all three PCR. In brief, nine percent (5/57) of mosquito pools were found positive. Among these five pools, three were *An. punctipennis*, one was *Ae. vexans,* and one was *Ur. sapphirina* pool. one positive pool from each of *An. punctipennis,* and *Ur. sapphirina* pool contained only male mosquitoes. The remaining pools were negative for *R. felis*. The 120-bp nucleotide sequences of the five mosquito pools positive in *R. felis*-specific *BioB*-based PCRs were identical to one another and to that of *R. felis* URRWXCal2 (CP000053.1). Among the 446-bp nucleotide sequences of the positive *gltA*-based PCRs, only a single base pair difference was found. These sequences were 99.7–100% identical to recognized *R. felis* strains in GenBank, and 84.0–95.7% identical to other *Rickettsia* spp. (**Figure 7**). The most closely related organisms were *Rickettsia lusitaniae, Rickettsia hoogstraalii,* and *Candidatus Rickettsia senegalensis* with which *R. felis* is known to cluster (Angelakis et al., 2016).

Discussion

Results of this study show that *R. felis* occurs in mosquitoes in the USA and adds *An. punctipennis, Ae. vexans* and *Ur. sapphirina* to the mosquitoes known to harbor the organism. It is noteworthy that one positive pool of *An. punctipennis,* one positive pool of *Ur. sapphirina* contained only male mosquitoes. As males do not take blood meals, it appears likely the infections were congenital and vertical transmission occurs in these mosquito species.

Opossums are the probable main mammalian reservoirs of *R. felis* in endemic areas in the USA (Anderson et al., 2019) while dogs have been implicated elsewhere in the world (Moonga et

al., 2019). None of the mosquitoes we found positive for *R. felis* have a reported tendency to feed on these species. *An. punctipennis* occurs widely in the eastern USA and feeds mostly on mammals, especially deer and sheep, but also on birds and people (Molaei et al., 2009). The cosmopolitan *Ae. vexans* feeds on people and other mammals, especially deer, cattle, horses, rabbits, sheep, and dogs (Greenberg et al., 2013). *Ur. sapphirina* is found in the eastern, central and southern US, and is the only mosquito known to feed on invertebrates, mainly earthworms and leeches (Reeves et al., 2018). Our finding that this species was infected with *R. felis* is of note as leeches have previously been suggested to be vectors of the organism (Slesak et al., 2015).

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Table 6 Primers and probes used for molecular detection of *Rickettsia felis*

PCR	Primer/probe	Nucleotide sequences (5'-3')	Amplicon	Reference
Rickettsia gltA FRET-qPCR	Forward-primer	TTRCAAATAGCAATAGAACTTGAAGCT	170 bp	(Zhang et al., 2014; Zhang, Lu, Kelly, et al., 2019)
	Reverse primer	AGCAAGAACCGTAGGCTGGAT		
	Anchor probe	ATCGCTCTTAAAGATGAATATTTTATTGAG-6-FAM-3		
	Reporter probe	LCRed640-GAAAATTATATCCAAATGTTGATTTTTATTC-phos		
Rickettsia gltA nested-PCR	Out-forward	AGTAAATCCAATAATAAAAAATGCKCTTAATA	446 bp	(Zhang et al., 2014; Zhang, Lu, Kelly, et al., 2019)
	Out-reverse	CTTAAAGATGAATATTTTATTGAGAGAAAAT		
	Inside-forward	ATGAGCAGAATGCTTCTACTTCAACA	353 bp	
	Inside-reverse	TTRCAAATAGCAATAGAACTTGAAGCT		
Rickettsia BioB-qPCR	Forward	ATGTTCGGGCTTCCGGTATG	120 bp	(Anderson et al., 2019)
	Reverse	CCGATTCAGCAGGTTCTTCAA		
	Probe	6-FAM-GCTGCGGCGGTATTTTAGGAATGGG-TAMRA		

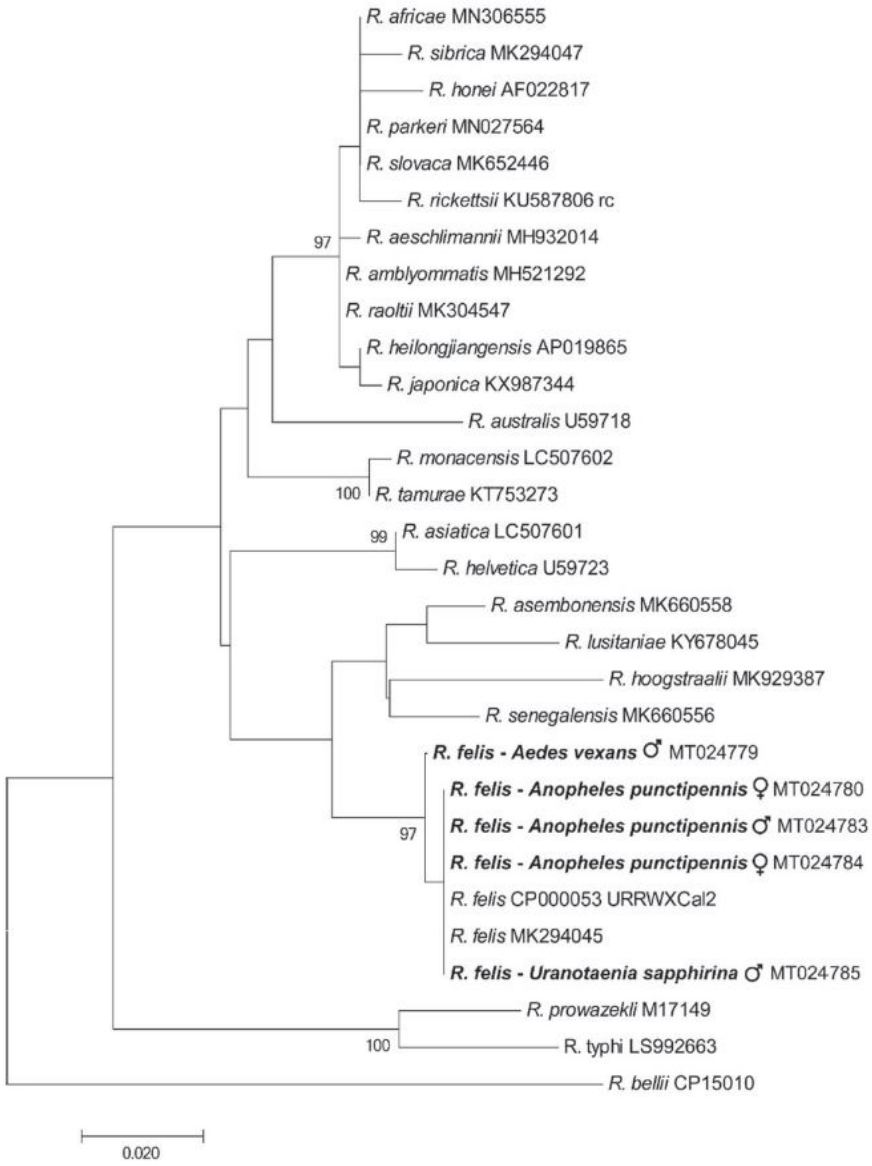

Figure 7 Phylogenetic tree using a bootstrap analysis for the *Rickettsia felis* found in mosquitoes from USA. The 446-bp nucleotide sequences of the *gltA* PCR products were concatenated and aligned using CLUSTALW, and the phylogenetic inferences were obtained from a maximum likelihood analysis. The names of Rickettsia species and their GenBank accession numbers are provided. The numbers at the nodes are the bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree. The sequences of *R. felis* identified in this study (in bold) were 99.7–100% identical to the recognized *R. felis* strains, and 84.0– 95.7% identical to other *Rickettsia* spp. The bootstrap values < 80 were omitted in the phylogenetic tree.

Chapter 4. Identification of *Rickettsia felis* DNA in the blood of domestic cats and dogs in the USA

Introduction

The intracellular bacterium *Rickettsia felis* is the agent of flea-borne spotted fever or cat-flea typhus (Hii, Kopp, Abdad, et al., 2011; Hii, Kopp, Thompson, et al., 2011), an emerging zoonotic disease. Since the first report in 1994 of *R. felis* in a person in Texas (Schriefer et al., 1994), infections have been described from around the world, with *R. felis* implicated as the causative agent of an important febrile illness in sub-Saharan Africa (Moonga et al., 2019a; Parola, 2011) and flea-borne spotted fever considered a global emerging threat to human health (Angelakis et al., 2016).

Various hematophagous arthropods, including a number of flea, tick, and mosquito species, have been found to carry *R. felis* (Angelakis et al., 2016; Higgins et al., 1994; Mediannikov et al., 2018; Philippe Parola et al., 2004), but *Ctenocephalides felis felis* (cat flea) is the only confirmed biological reservoir and vector of the infectious agent (Higgins et al., 1994). High percentages of cat fleas from around the world have been found to be infected with *R. felis*-for example, 30–79% in different U.S. states (Alabama, Maryland, Texas, Oklahoma, and Northern California) (Hawley et al., 2007; Noden et al., 2017; Stephenson et al., 2017). Experimental studies have shown that adult *C. felis felis* can become infected by feeding on rickettsemic animals, co-feeding with infected fleas, or as larvae feeding on infected adult feces, eggs, or other larvae (Ng-Nguyen et al., 2020; Reif & Macaluso, 2009). Infections are passed vertically and transstadially in subsequent generations of fleas, and *R. felis* is found in the salivary glands and be transmitted to mammalian hosts during feeding (Macaluso et al., 2008).

A number of mammals have been found to be PCR positive for *R. felis* DNA, mainly dogs (Moonga et al., 2019a), opossums (Panti-May et al., 2015; Schriefer et al., 1994), raccoons (Sashika et al., 2010), and rodents (Moonga et al., 2019a; Panti-May et al., 2015). Although cats were originally thought to be the mammalian reservoir hosts, experimental infections only revealed brief asymptomatic rickettsemia 2 months post infection, and then only in five of the 16 cats in the study (Wedincamp & Foil, 2000). Although naturally infected seropositive cats have been identified around the world (Reif & Macaluso, 2009), none have been found to be positive for *R. felis* in PCR assays performed on cats from Australia (*n*=11) (Barrs et al., 2010), China (*n*=135) (Zhang et al., 2014), Spain (*n*=212) (Segura et al., 2014), Thailand (*n*=585) (Assarasakorn et al., 2012; Phoosangwalthong et al., 2018), and the USA (*n*=121). Even cats infested with infected fleas are found to be PCR negative (Hawley et al., 2007; Segura et al., 2014; Zhang et al., 2014). In the USA, Stephenson et al. reported a *Rickettsia* seroprevalence of 3% in people, 42% in dogs, 79% in cats, 33% in gray foxes, and 83% in bobcats, but reverse transcription (RT)-PCR on blood tested consistently negative (Stephenson et al., 2017).

Several studies have shown that apparently healthy dogs are not uncommonly PCR positive for *R. felis*, with 3.5% of 200 owned dogs testing positive in Zambia (Moonga et al., 2019a), 9% of 100 pound dogs in Australia (Hii, Kopp, Abdad, et al., 2011), 2% of 130 indigenous community semi-domesticated dogs in Australia (Hii, Kopp, Thompson, et al., 2011), and a dog in the household of a Spanish flea-borne spotted-fever patient (Oteo et al., 2006). A recent study has shown experimentally infected dogs can be rickettsemic with *R. felis* for at least three months and be infectious for fleas, indicating they are a potential mammalian reservoir of the organism (Ng-Nguyen et al., 2020).

To shed further light on the *R. felis* status of dogs and cats from around the USA, we used PCR assays to analyze DNA from clinically ill cats and dogs from 45 states in the USA.

Methods and Materials

Whole blood samples

Whole blood samples in EDTA from 777 dogs from 45 states and 752 cats from 43 states of the USA were used in the study. These samples were submitted to the Molecular Diagnostics Laboratory at Auburn University College of Veterinary Medicine between 2008 and 2020. The canine blood samples were submitted for the molecular diagnosis of various tick-borne diseases (babesioses, hepatozoonoses, ehrlichioses, and anaplasmoses). Feline blood samples were submitted for the molecular diagnosis of FIPV (Feline Infectious Peritonitis Virus), FIV (Feline Immunodeficiency Virus), FeLV (Feline Leukemia Virus), and *Bartonella* infections. All the blood samples were sent to Auburn at ambient temperature.

DNA extraction

DNA was extracted from 800 µL aliquots of whole blood upon arrival of the samples using the High-Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions and following a similar protocol described before (Li et al., 2008; Poudel et al., 2020). The DNA was eluted in a 40 µL elution buffer. 20 µL volume of each DNA sample remaining after the tick-borne diseases (dogs) or FIP, FeLV, FIV, and *Bartonella* (cats) PCR assays had been performed was preserved at -80 ºC until the PCR assays to detect *R. felis* were performed in this study.

Detection of *Rickettsia* DNA by PCR

Cat and dog blood DNA samples were tested for *R felis* using citrate synthase (*gltA*)-based fluorescence resonance energy transfer (FRET), a nested PCR targeting the *gltA* of *Rickettsia*, and a *R. felis* species-specific *BioB*-based PCR (Anderson et al., 2019; Zhang, Lu, Li, et al., 2019). A list of the primers used in this study to detect *R. felis* is mentioned in **Table 6**.

Initially, 10 µL of each DNA sample was screened with the highly sensitive *Rickettsia* FRET qPCR. The remaining DNAs of the samples positive in the FRET-qPCR were arbitrarily diluted five-fold with 1× PBS, and 10-µL aliquots were used in the nested and *R. felis* species-specific *BioB*-based PCRs. The *R. felis* DNA from previous studies (Barua et al., 2020; Zhang, Lu, Li, et al., 2019) and nuclease-free water were used as positive and negative controls, respectively. The arbitrary fivefold dilution was also performed on the positive controls.

All PCR reactions were performed on a Roche Light Cycler 480 II thermocycler (Roche Diagnostics GmbH, Mannheim, Germany) as described above and previously in *Barua et al., 2020* (Barua et al., 2020). The products of *Rickettsia*-positive PCRs were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for Sanger DNA sequencing using upstream and downstream primers. The nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) to obtain GenBank Accession numbers, and a phylogenetic tree was generated to compare the nucleotide sequences of *Rickettsia* identified in this study with those of *R. felis*-like organisms (Odhiambo et al., 2014) and other *Rickettsia* species (**Figure 8**). The nucleotide sequences of the *gltA* PCR products were concatenated and aligned using CLUSTALW, and the phylogenetic inferences were obtained from a maximum likelihood analysis.

Results

The *Rickettsia* FRET-qPCR identified *Rickettsia* DNA in nine cats (1.2%) and eight dogs (1.0%). Of these 17 FRET-qPCR-positive samples, 8 were positive in the nested PCR assay (**Table 7**). Four of these DNA samples were also positive in the *R. felis*-specific *BioB*-based PCR assay.

Post PCR Sequencing of the nested PCR products amplifying the 300 bp *gltA* revealed that four cats (4/752, 0.53%) and three dogs (3/777, 0.39%) were positive for *R. felis* and one dog was positive for *R. rickettsii* (**Table 7**). Six (3 dogs and 3 cats) of these *gltA* nucleotide sequences were identical to the recognized *R. felis* type strain (CP00053 URRWXCaL2) (**Figure 8**) while one cat blood DNA had only a single nucleotide difference with the strain. The sequences of *R. felis* identified in this study had a lower similarity to the *R. felis*-like organisms (94.0% to *R. asembonensis*; 95.7% to *R. senegalensis*), and only 84.0–94.7% similarity to other *Rickettsia* spp. (**Figure 8**). The *R. felis*-specific *BioB*-based PCR assay was positive for all four of the *R. felis*-positive cat samples but was negative for the canine samples (**Table 7**).

Each of the four *R. felis*-positive cats was from different states: Kansas (sample was submitted in May 2013), California (July 2014), New York (July 2015), and Texas (November 2017). Three *R. felis*-positive dog samples were from Texas (November 2017) and Georgia (December 2014; December 2015), while the *R. rickettsii*-positive dog was from Alabama (June 2013). All three *R. felis* positive dogs were suffering from muscle and joint pain (**Table 7**).

Discussion

Recent reports of *R. felis* in mosquitoes (Barua et al., 2020) have raised the possibility of flea-borne spotted fever to be the next mosquito-borne pandemic (Parola et al., 2016). A better understanding of the mammalian hosts of *R. felis* bears importance in developing strategies to prevent and control potential outbreaks.

This study adds to existing data that *R. felis* occurs widely in the USA. These cats are referred by veterinarians for investigation for other suspected feline infections. Thus, these cats do not necessarily represent the actual cat population. Though very few cats (4/752) tested positive, the real scenario may be different. As cat fleas are very common, so the prevalence of *R. felis* may be much higher among cats in the USA. Moreover, these cats represent incidental cases that were naturally infected with *R. felis*. Dogs have been abundantly found seropositive while PCR positivity was less reported in different regions of the world (Hii et al., 2013; Hii, Kopp, Abdad, et al., 2011; Hii, Kopp, Thompson, et al., 2011; Stephenson et al., 2017; Zhang et al., 2014). However, it has recently been shown that dogs are reservoirs of *R. felis*, having asymptomatic rickettsemia for up to 100 days after experimental infection and being a source of infection for naïve fleas (Ng-Nguyen et al., 2020). The growing body of available data indicates that the importance of dogs in the epidemiology of *R. felis* might vary from area to area. To determine the role of dogs in the spread of *R. felis*, further studies are needed.

Although, this is the first description of naturally infected domestic cats that were PCR positive for *R. felis*, actually only very few animals (4/752) were found positive, which casts even further doubt on the possible role cats might play as reservoirs of *R. felis*. While the lower level of infections in cats might be because cats are less susceptible to infections, it might also be because cats are rickettsemic for very short periods before mounting immune responses that clear the infections (Wedincamp & Foil, 2000). In contrast, in dogs, circulating *R. felis* may persist for over three months (Ng-Nguyen et al., 2020), which would increase the likelihood of detection in random PCR surveys.

This study is the first to report copy numbers of *R. felis* DNA in dogs and cats. This study population consisted of cats and dogs that were clinically ill, with signs suggestive of tick-borne

diseases in the dogs and *Bartonella*, FIV, or FeLV infections in the cats. The fact that only relatively few of these clinically ill cats and dogs were PCR positive for *R. felis* is consistent with the existing data that infections are typically asymptomatic (Hii et al., 2013; Hii, Kopp, Abdad, et al., 2011; Hii, Kopp, Thompson, et al., 2011; Moonga et al., 2019b; Wedincamp & Foil, 2000, 2002) and that *R. felis* is an unlikely cause of fever in cats (Bayliss et al., 2009)

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Table 6. Primers and probes used for molecular detection of *Rickettsia felis*

PCR	Primer/probe	Nucleotide sequences (5'-3')	Amplicon	Reference
Rickettsia gltA FRET-qPCR	Forward-primer	TTRCAAATAGCAATAGAACTTGAAGCT	170 bp	(Zhang et al., 2014; Zhang, Lu, Kelly, et al., 2019)
	Reverse primer	AGCAAGAACCGTAGGCTGGAT		
	Anchor probe	ATCGCTCTTAAAGATGAATATTTTATTGAG-6-FAM-3		
	Reporter probe	LCRed640-GAAAATTATATCCAAATGTTGATTTTTATTC-phos		
Rickettsia gltA nested-PCR	Out-forward	AGTAAATCCAATAATAAAAAATGCKCTTAATA	446 bp	(Zhang et al., 2014; Zhang, Lu, Kelly, et al., 2019)
	Out-reverse	CTTAAAGATGAATATTTTATTGAGAGAAAAT		
	Inside-forward	ATGAGCAGAATGCTTCTACTTCAACA	353 bp	
	Inside-reverse	TTRCAAATAGCAATAGAACTTGAAGCT		
Rickettsia BioB-qPCR	Forward	ATGTTCGGGCTTCCGGTATG	120 bp	(Anderson et al., 2019)
	Reverse	CCGATTCAGCAGGTTCTTCAA		
	Probe	6-FAM-GCTGCGGCGGTATTTTAGGAATGGG-TAMRA		

Table 7. Identification of *Rickettsia* spp. in whole blood of cats and dogs by PCR assay

Sample ID	Host	State	Year	Background	FRET PCR	Nested PCR	*BioB* PCR
Ladyc	Cat	Manhattan, KS	May 2013	5-year; female, spayed; for *Bartonella*-negative	*R. felis*	*R. felis*	*R. felis*
IRBE32218089	Cat	Granada Hills, CA	July 2014	5-year; male castrated; Siamese; for FIP-negative	*R. felis*	*R. felis*	*R. felis*
Clara	Cat	Brooklyn, NY	Nov 2015	American domestic shorthair; 8-month; female; for FIPnegative	*R. felis*	*R. felis*	*R. felis*
Fritz	Cat	Spring, TX	2017	7-month; American domestic shorthair; male, castrated; for FIP-negative	*R. felis*	*R. felis*	*R. felis*
Rebar	Dog	Montgomery, AL	June 2013	Male; for *Hepatozoon*-negative	*R. rickettsii*	*R. rickettsii*	Failed
Daisy	Dog	Killeen, TX	Nov 2017	Two-month shifting leg lameness. Jack Russell Terrier Mix; female, spayed; 6Y. For Hepatozoon-negative	*R. felis*	*R. felis*	Failed
Roxie	Dog	Jefferson, GA	Dec 2014	Bulldog; female; 6-year; chronic weight loss with muscle/joint pain; for *Hepatozoon*-negative	*R. felis*	*R. felis*	Failed
Kawlija	Dog	Rome, GA	Dec 2015	Jack Russell Terrier; 11-year; male; for *Hepatozoon*-negative; 6-week history of vomiting/diarrhea; intermittent pain	*R. felis*	*R. felis*	Failed

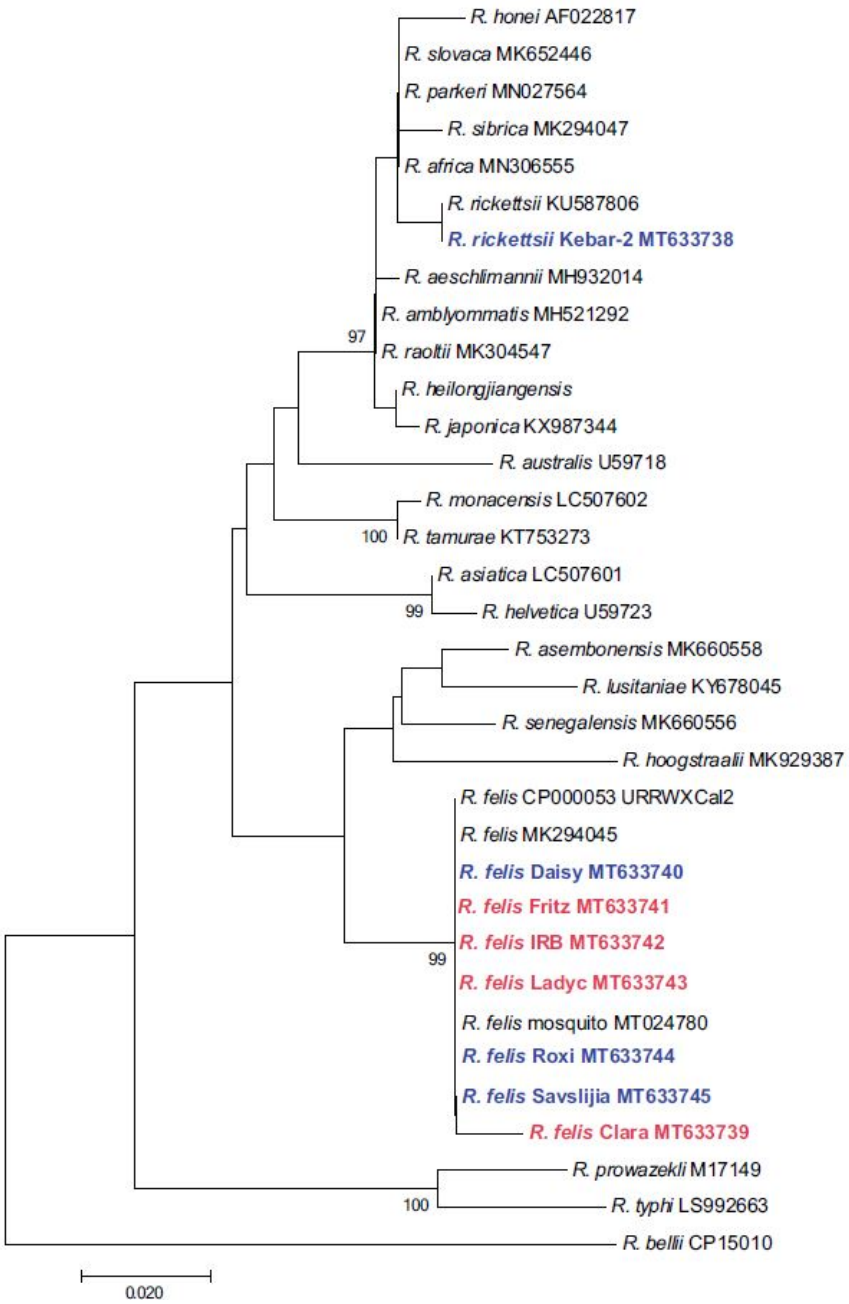

Figure 8 Phylogenetic tree using a bootstrap analysis for the *Rickettsia felis* found in cat and dog blood from the USA. The names of Rickettsia species and their GenBank Accession numbers are provided. The sequences of *R. felis* identified in this study (in bold; red for cats and blue for dogs) were 99.0–100% identical to the recognized *R. felis* strains, 94.0% (*R. asembonensis*) and 95.7% (R. senegalensis) identical to *R. felis*-like organisms, and 84.0–94.7% identical to other *Rickettsia* spp.

Chapter 5. Conclusion and future research

The papers included in the present thesis have detected *Rickettsia felis* in mosquitoes and in cats and dogs of the USA, which points towards the potential of dissemination of this pathogen in this region and adds supporting evidence to the rising incidence of flea-borne spotted fever. Along with this, the newly developed and validated primer system mentioned here provides a more accurate DNA barcoding system for the identification of mosquito species.

An appropriate level of control measures, a minimum level of vaccination coverage is required per the epidemic threshold to prevent or control an epidemic focus. However, *Rickettsia felis* infection might go unnoticed and trigger a wave of a massive outbreak because it causes unrecognized febrile illness and its clinical features mimic a variety of other infections. It is already becoming more common in sub-Saharan Africa since the first report about 2 decades back. There are still knowledge gaps regarding its transmission, pathophysiology, mammal hosts, vector competence, and capacity, which need more epidemiological and animal model studies to introduce.

Mosquito species complex, cryptic species, and accurate identification is a constant challenge in the mosquito research field in terms of time, resources, and expertise. Endemic mosquito species might turn out to be efficient vectors of a new pathogen, or endemic pathogens might be efficiently transmitted by imported mosquito species. Mosquito-borne diseases are linked to the presence of vector-competent mosquito species and occur in regions where temperatures allow for the completion of the extrinsic development of the pathogens. So, it is not a problem confined only to the tropics. Mosquito vector research on indigenous species should be further intensified to prepare well-founded risk assessments of outbreaks of mosquito-borne diseases. As climate change and global transportation are getting more intense, vectors and pathogens are

spreading beyond their native ranges. Cats being the natural host of *Rickettsia felis*'s ubiquitous vector, cat flea and mosquitoes being an important vector for this pathogen, animal model and vector transmission studies should be designed with them.

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