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Transmission dynamics of *Bartonella* spp. in cave-dwelling bats and bat flies in Costa Rica

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By

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Abstract

Transmission dynamics of *Bartonella* spp. in cave-dwelling bats and bat flies in Costa Rica

By Miranda Mitchell

Purpose: This thesis investigated the transmission dynamics of *Bartonella* spp. in Costa Rican bats and bat flies. The thesis aimed to increase scientific understanding of the prevalence of *Bartonella* in bat and bat flies, as well as assess spillover risk to humans.

Methods: In 2018, 294 individual bats (n=18 species) were sampled using hand nets across 15 roosts throughout the southern and northwestern regions of Costa Rica. Blood samples were obtained from 258 bats and 114 ectoparasites were collected from 48 bats, following an approved IACUC protocol (DAR-4000049-ENTRPR-N). Bat flies were identified taxonomically and pooled by individual bat host and bat fly species (n=63 pools), and DNA was extracted from blood and ectoparasite samples. Samples were screened for *Bartonella* via polymerase chain reaction (PCR) targeting the *gltA* citrate synthase gene. PCR-positive samples were sequenced. Phylogenetic trees comparing these isolates to previously identified Central American and globally named strains were stratified by country and constructed using Bayesian MCMC analyses, executed by MrBayes 3.2.6. A complete tree was constructed with 10,000,000 generations and a burn-in fraction of 25%.

Results: *Bartonella* PCR-prevalence from all samples was 14.6% (47/321). *Bartonella* PCR-prevalence was 10.4% (27/258) for bats and 31.7% (20/63) for ectoparasite pools. *Bartonella* isolates from bats (n = 8) and bat flies (n= 5) were included in phylogenetic analyses, which revealed 11 genetic variants, including four newly described genotypes. These 11 genetic variants clustered into nine clades of 96.0%-99.2% similarity. Bat and bat fly genotypes from this study clustered with previously identified *Bartonella* sequences from bats and bat flies from Belize, Guatemala and Costa Rica. Four clades were unique to this study.

Conclusions: This thesis expanded upon existing knowledge of the diversity and prevalence of *Bartonella* in Costa Rican bats and bat flies. *Bartonella* were more prevalent in bat flies than bats. Identical *Bartonella* strains were found in bats and bat flies, suggesting potential for sharing of the pathogen. Geographic and host associations were observed among *Bartonella* strains from bats and bat flies from Central America; however, significant areas of overlap were also observed.

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1. Introduction

1.1 Overview & Significance

Emerging infectious diseases (EIDs) are a major concern in public health. As many as 75% of EIDs are thought to be zoonoses, or diseases transmitted from animals to humans (Centers for Disease Control and Prevention, 2018). Bats are important potential reservoirs for zoonotic emerging and re-emerging infectious diseases (Calisher *et al.* 2006; Turmelle and Olival 2009), whose diets, high mobility, broad geographic distribution, seasonal migration, patterns of daily movement, long life-span, and unique social behaviors (i.e., communal roosting and fission-fusion social structure) enhance their reservoir capacity (Bai *et al.* 2011; Calisher *et al.* 2006). Consequently, recent studies have aimed to investigate the potential for bats to serve as reservoirs for bacterial pathogens that may cause EIDs, such as *Bartonella* spp. (Judson *et al.* 2015; Veikkolainen *et al.* 2014; Mühldorfer 2013; Wood *et al*. 2012).

Bartonella is a genus of gram-negative bacteria that can act as opportunistic pathogens (Harms and Dehio 2012) and *Bartonella* spp. have been identified as etiologic agents for zoonotic diseases (Judson *et al.* 2015; Morse *et al.* 2012; Bai *et al.* 2011). Humans are considered incidental or dead-end hosts for several *Bartonella* spp. and therefore often only become infected as a result of exposure to infected animals or arthropods (Judson *et al.* 2015). However, *Bartonella* spp. have been associated with several human illnesses and are responsible an increasing amount of EIDs, including a neglected tropical disease (NTD) called bartonellosis (Bai *et al.* 2012; Kosoy *et al.* 2008; Kosoy *et al.* 2003; Roux *et al.* 2000; Eremeeva *et al.* 2007; Kerkhoff *et al.* 1999; Welch *et al.* 1999; Bass *et al.* 1997; Daly *et al.* 1993; Welch *et al.* 1992). Bartonellosis can cause

a gamut of symptoms including severe diseases such as endocarditis, lymphadenitis, and meningitis (Morse *et al.* 2012). Past human bartonellosis outbreaks include Trench Fever, caused by *Bartonella quintana,* and cat scratch fever, caused by *Bartonella henselae* (Harms and Dehio 2012). *Bartonella bacilliformis* is also known to cause another febrile illness called Carrion's disease (Minnick *et al.* 2014). These *Bartonella*-caused symptoms and diseases can be acute, subacute, chronic and near fatal. The transmission dynamics of *Bartonella* spp. in and between mammalian hosts is understudied; however, bats and arthropods such as fleas, flies, mites and ticks are often implicated as reservoirs and vector species, respectively (Brook *et al.* 2015; Morse *et al.* 2012).

In 2012, Morse *et al.* found evolutionary association between bartonellae and blood-feeding ectoparasites, such as bat flies, through phylogenetic and gene network analyses (Morse *et al.* 2012). Bat flies are obligate blood-sucking ectoparasites that parasitize bats, living in their fur and on their wings (Dick & Patterson 2006; Dick and Dittmar 2013). Taxonomically, these flies fall into two general families: the Nycteribidae and the Streblidae. The Nycteribidae resemble spiders and have no wings, whereas the Streblidae resemble other dipterans and generally have full or reduced wings and sometimes no wings at all. However, bat flies have diverse morphologies, even within these families. Moreover, like other flies, bat flies are holometabolous and need to metamorphose (Patterson *et al.* 2007). Thus, the pupation stage for bat flies occurs off their bat hosts, somewhere in the bats' roost (Ibid; Fritz 1983; Ross 1961). After this occurs, bat flies must move to a new bat host for a blood-meal (Ibid; Caire *et al.* 1985). Although this developmental transition theoretically may allow bat flies to "jump" between species, most bat fly species are thought to be host-specific and are often

recorded from the same species of bat (Ibid; Dick & Gettinger 2005; Wenzel 1976). When bat flies are found parasitizing two or more bats, they are often found on bat host species within the same genus (Ibid; Patterson *et al.* 1998, ter Hofstedete and Fenton 2005). The host-specific parasitism of these flies may suggest that bat species and their associated bat fly species are more likely to share strains of *Bartonella* spp., presenting a potential mode of transmission between an arthropod vector, the bat fly, and a reservoir host, the bat.

Caves are optimal roosts for bats. These roosts shield bat populations from inclement weather, protect them from their predators, and provide them with a centralized place for social interactions. Caves are a roost type of interest for public health because these habitats allow for diverse bat species to interact in proximity, thus potentially facilitating the sharing of pathogens among species. Therefore, to better understand the transmission dynamics of *Bartonella* in Costa Rican bats and their bat flies, this study investigated *Bartonella* infection specifically in cave-dwelling bats and bat flies in Costa Rica. Ultimately, investigating the transmission dynamics of bacterial pathogens such as *Bartonella* in arthropods, wildlife, domestic animals and humans is significant to tropical medicine, veterinary science and public health. This investigation aims to shed light on *Bartonella* strains in wild Costa Rican bat and the associated bat fly community, which can lead to greater scientific understanding of this pathogen's potential threat to animal and human populations. Furthermore, knowledge of the diversity and prevalence of potentially pathogenic bartonellae in bats and bat flies can lead to the establishment of best practices for biologists working with bats, members of the public who may interact with these animals, and physicians treating and diagnosing febrile illness.

1.2 Background: *Bartonella* species in bats and bat flies in Central America

A series of studies have reported *Bartonella* infection in bats and bat flies globally. *Bartonella* infection has been recorded in bats in Belize, China, Costa Rica, Finland, France, Ghana, Guatemala, Kenya, Madagascar, Nigeria, Panama, Peru, Republic of Georgia, Spain, Tanzania, Uganda, the UK, among other locations. (Becker *et al.* 2018; Urushadze *et al.* 2017; Han *et al.* 2017; Stuckey *et al.* 2017; Lilley *et al.* 2015; Judson *et al.* 2015; Bai *et al.* 2011; Kosoy *et al.* 2010; Veikkolainen *et al.* 2014; Brook *et al.* 2015; Mannerings *et al.* 2016; Kamani *et al.* 2014). This wide global distribution, as well as *Bartonella*'s phylogenetic complexity, has led to increasing popularity in studies focused on the host-vector dynamics of *Bartonella*, as well as molecular characterization of this bacteria in bats and bat flies (Kosoy *et al.* 2017).

Since the prevalence and spillover risk of EIDs such as those caused by *Bartonella* spp. are hypothesized to increase with anthropogenic disturbance (i.e., land-use change, habitat fragmentation and deforestation) and tropical climates most threatened by this anthropogenic disturbance are thought to accommodate the majority of emerging infectious diseases, it is important to investigate *Bartonella* infections in natural systems within neotropical Central America (Gillespie *et al.* 2005; Gillespie and Chapman 2006; Cottontail *et al.* 2009; Morse 1995; Patz *et al.* 2000). Narrowing the lens to this region, *Bartonella* spp. in bats has previously been described in Guatemala, Belize, Panama and Costa Rica. In 2011, Bai *et al.* collected blood specimens from 118 bats, representing 10 genera and 15 species from five independent sites in Southern Guatemala (Bai *et al.* 2011). These blood samples were used for *Bartonella* culture, resulting in 33.1% of bat samples yielding *Bartonella* isolates. The bat species that were

positive for *Bartonella* isolates included: one (5.9%) Neotropical fruit bat (*Artibeus* spp.), one (8.3%) Little yellow-shouldered bat (*Sturnira lilium*), four (28.6%) Seba short-tailed bats (*Carollia perspicillata*), two (13.3%) Pallas's long-tongued bats (*Glossophaga soricina*), seven (70%) lesser naked-backed bats (*Pteronotus fulvus*), 15 (48.4%) common vampire bats (*Desmodus rotundus*), one (33.3%) common big eared bat (*Micronycteris microtis*), and eight (88.9%) pale spear-nosed bats (*Phyllostomus discolor*) (Bai *et al.* 2011; Stuckey *et al.* 2017). Isolates were confirmed by PCR using primers BhCS781.p and BhCS1137.n to target the citrate synthase gene (*gltA*). Through phylogenetic analysis, the authors identified 21 genetic variants of *Bartonella*, which clustered into 13 phylogroups. Each phylogroup represented between one and six genotypes, all of which had 96.2-99.7% sequence identity. Some groups in phylogenetic analysis included isolates that originated in a variety of different, diverse bat species. These patterns in grouping may suggest that different bat hosts may harbor and share strains of *Bartonella*.

In another study from Guatemala, Wray *et al*. focused only on the common vampire bat, sampling 103 individuals, producing 396 blood, urine, saliva and fecal samples (Wray *et al.* 2016). *D. rotundus* is a host-reservoir of high concern for studies of blood-borne pathogens because they rely only on blood as a food source. Although Wray *et al*.'s PCR assay for *Bartonella* targeting the *gltA* gene was unsuccessful, the researchers conducted a secondary PCR assay to target a different gene commonly used to identify *Bartonella* spp., *ribC,* using primers BARTON-1 and BARTON-2 from Johnson *et al.* 2003 (Wray *et al.* 2016; Johnson *et al.* 2003). The *ribC* assay revealed that 43 (10.9%) bat samples were positive for *Bartonella* across a range of sample types. The

majority, 35 (39.3%) were positive blood clots, but five (4.9%) fecal swabs and three (3.4%) serum samples were positive as well (Wray *et al.* 2016; Stuckey *et al.* 2017). No oral swab or urine samples generated positive results. These 43 positive samples came from 39 individual bats. Thus, these authors reported an overall *Bartonella* PCR-prevalence of 37.9%. In their discussion, Wray *et al.* suggest potential for inter-species transmission of these *Bartonella* from *Desmodus rotundus,* bat fly ectoparasites, and other bats*,* especially considering this study's positive fecal swabs and their additional investigation into prey preference using Cytochrome B analysis. However, these authors note that inter-species transmission is largely uncharacterized in the literature. These studies in Guatemala further emphasize the need for follow-up investigations into possible spillover risk of pathogenic *Bartonella* from bats to domestic animals.

In a study conducted in 2012 and 2013, bats and their bat flies were captured in mist nets across 18 sampling sites in southern Costa Rica. Bat blood was filtered and represented 63 individuals from 22 bat species. These blood samples were tested for *Bartonella* using a PCR assay that targeting the *gltA* gene with primers 443f and 1210r. The results of this study yielded positives across 13 bat species: one (16.6%) Chestnut short-tailed bat (*Carollia castanea*), one (33.3%) Talamancan yellow-shouldered bat (*Sturnira mordax*), two (28.6%) little yellow-shouldered bats (*Sturnira lilium*), one (33.3%) pale spear-nosed bat (*Phyllostomus discolor*), three (50%) great fruit eating bats (*Artibeus literatus*), two (33.3%) Seba's short-tailed bats (*Carollia perspicillata*), one (50%) greater broad-nosed bat (*Platyrrhinus vittatus*), two (50%) Geoffroy's tailless bats (*Anoura geoffroyi*), one (50%) Jamaican fruit bat (*Artibeus jamaicensis*), one (50%) northern little yellow-eared bat (*Vampyressa thyone*), three (75%) hairy-legged myotis

(*Myotis keaysi*), two (66.6%) Sowell short-tailed bats (*Carollia sowelli*) and one (100%) common big-eared bat (*Micronycteris microtis*) (Judson *et al.* 2015; Stuckey *et al.* 2017).

This study from Costa Rica also tested 55 bat fly individuals, obtained from bats sampled in the field. Bat flies had a prevalence of 52.7% (29/55) for Bartonella. Every bat fly species that was positive for *Bartonella* infection had a new association with the bacteria. For the bats who were parasitized by bat flies during sampling, one individual bat fly was used as a representative of each species and was tested for *Bartonella,* resulting in analysis of 44 host-vector pairs. In 12 of the 44 pairs (27.2%), both the bat and bat fly were positive for *Bartonella*. In three (6.8%) of the 44 pairs, only the bat host was positive for *Bartonella*. In 13 (29.5%) of the pairs, only the ectoparasite was positive for *Bartonella*. The authors conclude that bat flies were more likely to be PCR-positive for *Bartonella* than their bat host counterparts. The authors conducted global phylogenetic analysis with previously identified *Bartonella* species, as well as previously described *Bartonella* species genetic variants from Guatemala, Peru, Panama and Puerto Rico. Their analysis revealed 25 newly described *Bartonella* variants out of 27 total *Bartonella* variants. These 25 newly described variants clustered into a total of 20 clades, which were all between 96-99.7% sequence identity. Only one Costa Rican genetic variant (GenBank accession number KJ816674), from an *Aspidoptera delatorrei* bat fly from a *Sturnira lilium* bat, was the same genetic variants as a variant identified in a Guatemalan *Carollia perspicillata* bat (HM597199). Additionally, one isolate derived from a Costa Rican *Trichobius joblingi* bat fly from a *Carollia perspicillata* bat (KJ816691) was identical to an isolate found in a Peruvian *Carollia perspicillata* bat (JQ071386), thus representing identical strains from a specific bat host species and a bat

fly from that bat host species between countries. Although newly described, four ectoparasite variants also grouped in the same clade with variants previously detected in Guatemalan bats. These variants from Costa Rican ectoparasites and Guatemalan bats were from diverse species of bats and bat flies, respectively. None of the Costa Rican variants from this study fell into clades with strains from Mexican, Panamanian, Puerto Rican or African bat flies or African bats.

In 2018, a study published by Becker *et al*. documented *Bartonella* infection prevalence across 193 common vampire bats from Belize, Guatemala, Mexico and Peru, to be 67% (Becker *et al*. 2018). These authors conducted global phylogenetic analysis of 35 common vampire bat *Bartonella* isolates, which had 78.8-100% pairwise identity in the *gltA* gene. This analysis identified 11 para-phyletic genotypes. The authors also used a software package called BaTS, which allows users to test for significant phylogeny-trait correlations, which are "defined as the extent to which neighboring taxa in a phylogenetic tree share a character of interest" (Parker *et al.* 2008). These characters may be a phenotypic trait, molecular marker or geographic characteristic. The package tests for phylogeny-trait correlations while addressing uncertainty from phylogenetic error by integrating credible topologies produced from Bayesian phylogenetic analysis programs such as MrBayes or BEAST. The BaTS analysis of the *Bartonella* isolates associated with vampire bats revealed significant phylogenetic clustering by country, although most of the genotypes were still widely distributed. Two genotypes were detected across all regions, and four were detected in both Belize and Peru, which suggests a broad distribution of vampire bat *Bartonella* genotypes. Regardless, one genotype was found to be unique to Peru, three were unique to the Peruvian Amazon and one was unique to

Belize. The phylogenetic position of the common vampire bat *Bartonella* sequences was assessed among known *Bartonella* genotypes. Approximately one half of the Belizean and Peruvian sequences (18/35) were almost identical (>99.7% sequence identity) to isolates of *Bartonella* from Mexican common vampire bats, which also confirmed the wide geographic distribution of genotypes. Nine other sequences fell within the same clade (>96% pairwise identity) as *Bartonella* from Panamanian bat flies (*Strebla diaemi*), including isolates from the mustached bat (*Pteronotus mesoamericanus*) in Mexico, from Seba's short-tailed bats in Peru (*Carollia perspicillata*) and Pallas's long-tongued bat in Guatemala (*Glossophaga soricina*), or from the aforementioned Mexican vampire bats. In my study, these bat species were observed to commonly roost with common vampire bats at the sampled sites (Table 1). This suggests that the genotypes of *Bartonella* shared among species of bats and bat flies may be reflective of species roosting patterns. Of the isolates from this study, eight were considered novel, meaning that they had less than 96% identity to GenBank sequences. However, these sequences were most like *Bartonella* from the Mexican vampire bats and Costa Rican bats (*Anoura geoffroyi*).

Other studies have identified bartonellae in Central American bat flies. In a study of the genetic diversity and overall global distribution of *Bartonella* in bat flies, Morse *et al.* reported *Bartonella* spp. in bat flies in Panama and Costa Rica (Morse *et al*. 2012). Additionally, other studies have documented high *Bartonella* prevalence in bat flies from Panama, Guatemala and Costa Rica as well (Judson *et al*. 2015, Becker *et al*. 2018). The establishment of bartonellae in bats and associated bat flies confirms the potential for bat flies to serve as vectors of the bacteria and suggests a possible mechanism for transmission between and among bats (Morse *et al.* 2012; Billeter *et al.* 2012).

Even though some samples from Becker *et al*.'s study originated from cave roosts, to my knowledge, bats and bat flies sampled in the previous Costa Rican and Guatemalan studies were not collected within cave roosts. My study aims to contribute to the literature on this subject by sampling bats within their cave roosts and across diverse habitats throughout the country of Costa Rica. Sampling within cave roosts is important for investigating the bat-bat fly relationship because "cavity-roosting" (i.e., bats roosting in man-made or natural caves) bat species have been shown to have higher densities of ectoparasites (both bat flies and mites) compared to non-cavity roosting bats (ter Hofstede and Fenton 2005). Moreover, intraspecies and interspecies transmission potential of *Bartonella* infection may be higher in caves, which are habitats in which many bat species can roost together and reach high population densities (Bai *et al.* 2011). Caves are also fragile habitats that may be more vulnerable to anthropogenic disturbance, which could lead to increased stress among bat populations, lowered immune function and thus higher pathogen prevalence.

Notably, previous studies have focused sampling efforts in independent sites in the same general region, representing some tropical wet forest and farmland. Sampling across many roosts located in diverse habitat types, including a gradient of tropical wet forest, dry forest and agricultural pastures, and regions throughout Costa Rica would contribute an important landscape component to scientific understanding of the prevalence and diversity of *Bartonella* species in bats and bat flies.

1.3 Goals, Questions & Hypothesis

The goals of this study were to: (1) increase and expand existing scientific understanding of the prevalence of *Bartonella* spp. in bat and bat fly samples across a diverse range of Costa Rican cave environments, thus contributing a new roost-focused perspective to the literature on this subject, and (2) assess relationships among *Bartonella* strains identified in humans, domestic animals, and those identified in bats and bat flies through phylogenetic analyses. My question is: What *Bartonella* strains are being shared in humans, domestic animals, bats and bat flies? Lastly, this study was hypothesis-generating and did not test any specific hypotheses. These goals and question were addressed through molecular and phylogenetic analyses.

2. Materials and Methods

2.1 Study Design, Methods of Data Collection and Study Sites

Bats were captured using hand nets during 2018 across 15 roosts throughout the southern and northwestern regions of Costa Rica (Figure 1). The cave locations represent a gradient of tropical wet forests, dry forests and farmland. Sample sites included natural caves and man-made roost sites (mines and tunnels). The sites are located in the Chorotega Region (Santa Rosa National Park (cave El Duende), Barra Honda National Park (caves Pozo Hediondo and Ramón Canela, which were clustered for analyses due to their proximity), and Rincón de la Vieja National Park (cave Los Araya), as well as Cave Venado in Gabinarraca, and Tunel Arenal in the farmland), the Central Pacific Region (Absolute Reserve Cabo Blanco (caves El Peñón y la Grande, which were also clustered) and cave Damas, in Quepos), the Brunca Region (Piedras Blancas National Park (cave Laguna Perdida), cave

Corredores and cave Emus, and Túnel ICE 2 (man-made)), the Central Region (mine El Aguacate, cave Mastatal, cave Locos por el Bosque (Biological Reserve in Monteserrat de Coronado) and Tres Ríos, a testing site for sampling that was not a man-made or natural roost).

Bats were caught, weighed, measured, sexed and identified by bat species based on morphology and released after blood and parasite sample collection. After taking measurements of each bat, blood samples and ectoparasites were taken from bats, following our approved IACUC protocol (DAR-4000049-ENTRPR-N). Blood was stored in Eppendorf tubes with RNAlater and bat flies were collected from bats' fur and wings using forceps and placed in 96% ethanol. Ectoparasite and blood samples were stored at -20C until used for DNA extraction. Bat flies were identified using Wenzel's taxonomic keys in *Ectoparasites of Panama* and the online key, "The Bat Flies of La Selva," available at http://www.biologie.uni- ulm.de/bio3/Batfly/index.html (Wenzel and Tipton, 1966; Tschapka & Miller, 2009). A stereoscope was used to identify individual bat flies to species, which were grouped in pools based on bat host species and bat fly species identification and then extracted in these pools.

2.2 Molecular Analysis

Molecular laboratory methods were used to determine the presence of *Bartonella* DNA in bat and bat fly blood and whole insect samples. DNA was extracted from 41 blood samples using the Macherey-Nagel NucleoSpin DNA Blood kit and extracted from 114 ectoparasite samples using the Macherey-Nagel NucleoSpin DNA Insect kit at Emory University. 217 blood samples were extracted at the University of Costa Rica in San Jose

using the Thermo Fisher Scientific PureLink Genomic DNA kit. DNA extractions were stored in Eppendorf tubes in a -80C freezer at Emory University for long-term use, as these samples were also used as a part of a larger study investigating other parasites and pathogens such as *Trypanosoma* spp. and *Leptospira* spp.

Extracted DNA was screened for *Bartonella* spp. via polymerase chain reaction (PCR) to amplify a 770 bp portion of the partial citrate synthase gene (*gltA*) using previously published primers 443f (5′ GCT ATG TCTGCA TTC TAT CA 3') (Birtles and Raoult, 1996, as cited in Billeter *et al.* 2012) and 1210r (5′ GAT CYT CAA TCA TTT CTT TCC A 3') (Billeter *et al*. 2012). The initial PCR screen used a modified 12.5µL PCR protocol adapted from a modified 10 µL from Judson *et al*., followed by a modified 25µL confirmation PCR for *Bartonella* positive samples (Judson *et al.* 2015). Each reaction contained 10µM of each primer, 1x Taq PCR Master Mix (Qiagen), nuclease-free water and genomic DNA. Reactions were performed using a Mastercycler Pro thermal cycler (Eppendorf) using the following cycling conditions: 94°C for 2 min followed by 45 cycles of 94°C for 30 sec, 48°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 7 min (Billeter *et al.* 2012). A positive control of *Bartonella doshiae*-DNA, donated by Dr. Michael Kosoy and Dr. Maria Rosales Rizzo from the Centers for Disease Control and Prevention and a negative control of nuclease-free water were used in each PCR assay.

To determine the accurate amplification and presence or absence of *Bartonella* DNA in samples, 5uL of PCR products were visualized using 1.5% agarose gels. Gels were stained with Gel Red Nucleic Acid (Biotium).

2.3 Sequencing and Phylogenetic Analyses

Amplicons that displayed strong enough bands in the confirmation PCR to be sent out for sequencing by Macrogen Inc. Forward and reverse sequences were assembled into contigs and consensus sequences using De Novo assembly in the software package Geneious Prime created by Biomatters, available from http://www.geneious.com/. For samples B4, B16 and BE1 individual strands (forward or reverse) were used due to the significantly higher quality of these individual strands than their complements. The amplification of *Bartonella gltA* was confirmed by comparing the sequenced PCR products to sequences in GenBank.

To create a global phylogeny, the consensus sequences were aligned to 22 *Bartonella* sequences isolated from Guatemalan bats and bat flies, 13 *Bartonella* sequences from common vampire bats from Belize, 2 *Bartonella* sequences isolated from Panamanian bats and bat flies, as well as 27 previously described *Bartonella* sequences from bats and bat flies in Costa Rica and 23 globally described *Bartonella* spp. (Tables 4 & 5) (Bai *et al*. 2012, Judson *et al*. 2015). 770bp consensus sequences from Costa Rican bat and bat fly samples were trimmed to approximately 250bp for alignment to global reference sequences in GenBank, which were approximately 300bp in length.

Brucella melitensis was used as an outgroup to root the tree (Morse *et al.* 2012; Judson *et al.* 2015). The alignment was created using the multiple alignment program MAFFT. The L-INS-i refinement method was used to create a more accurate alignment.

The alignment was manually checked in the MEGA X software. It was then further refined with the alignment refinement tool, Gblocks, available at http://molevol.cmima.csic.es/castresana/Gblocks.html, which eliminates poorly aligned and its suitability for phylogenetic analysis. Phylogenetic trees comparing 2018 Costa Rican

isolates to previously identified Central American identified strains were stratified by country: Costa Rica 2018 from this study only, Costa Rica 2015 and 2018, Costa Rica 2018 and Belize, Costa Rica 2018 and Guatemala, Costa Rica 2018 and Panama. Additionally, trees were constructed comparing Costa Rica 2018 isolates to 21 globally identified *Bartonella* species. A full tree including all sequences was included as well. Sequences with >96.0% sequence similarities were considered part of one shared clade, since 96.0% identity in the *gltA* gene has regularly been used as the cut-off for the identification of *Bartonella* species (Scola *et al.* 2003; Judson *et al.* 2015). All trees were constructed using Bayesian MCMC analyses, executed by MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001). Stratified trees were constructed with 100,000 generations and a burn-in fraction of 25%. The complete tree was constructed using MrBayes with 10,000,000 generations and a burn-in fraction of 25%. The parameters for the nucleotide changes were determined using JModelTest v.2.0 (Posada, 2008) using the maximum likelihood method.

3. Results

3.1 Sample Size

Two-hundred and ninety-four individual bats from 18 species were sampled across 15 sites. Blood samples were taken from 261 bats and 114 ectoparasite samples were taken 48 bats. The 114 ectoparasite samples were pooled by bat fly species per bat host sampled, yielding 63 ectoparasite species pools, representing 23 bat fly species.

Bat species (n=18) sampled at these sites included: *Anoura cultrata* (n=2), *Artibeus jamaicensis* (n=3), *Balantiopteryx plicata* (n=5), *Carollia perspicillata* (n=94), *Desmodus rotundus* (n=26), *Diphylla ecaudata* (n=1), *Glossophaga commisarisi* (n=12),

Glossophaga soricina (n=11), *Lonchophylla robusta* (n=26), *Lonchorhina aurita* (n=14), *Macrophyllum macrophyllum* (n=2), *Phyllostomus hastatus* (n=9), *Pteronotus gymonotus* (n=15), *Pteronotus mesoamericanus* (n=63), *Pteropteryx kappleri* (n=1), *Saccopterx bilineata* (n=1), *Tonatia saurophila* (n=1) and *Trachops cirrhosus* (n=8).

Bat fly species (n=23) sampled from these bats included: *Aspidoptera phyllostomasis* (individual n=3; pool n=1), *Exastinion clovisi* (individual n=3; pool n=2), *Megistopoda aranea* (individual n=5; pool n=4); *Speiseria ambigua* (individual n=5; pool n=1); *Strebla carolliae* (individual n=1; pool n=1); *Strebla diaemi* (individual n=1; pool n=1); *Strebla galindoi* (individual n=3; pool n=2); *Strebla guajiro* (individual n=1; pool n=1); *Strebla hertigi* (individual n=2; pool n=1); *Strebla mirabilis* (individual n=1; pool n=1); *Strebla vespertilionis* (individual n=9; pool n=2); *Trichobius dunni* (individual n=2; pool n=1); *Trichobius caecus* (individual n=3; pool n=3); *Trichobius furmani* (individual n=1; pool n=1); *Trichobius yunkeri* (individual n=4; pool n=3); *Trichobius dugesiodes* (individual n=2; pool n=2), *Trichobius galei*, (individual n=3; pool n=3), *Trichobius johnsonae* (individual n=9; pool n=3), *Trichobius keenani* (individual n=3; pool n=1), *Trichobius pallidus* (individual n=46; pool n=22), *Trichobius perspicillatus* (individual n=1; pool n=1), *Trichobius sparsus* (individual n=3; pool n=3), *Trichobius uniformis* (individual n=3; pool n=2). A new pool of each species indicates that this ectoparasite species was sampled from a different individual bat.

3.2 PCR Prevalence

Bartonella species were detected in 27 out of 258 individual bats (14.6%) as well as 20 out of 63 ectoparasite pools (31.1%). PCR *Bartonella* prevalence among bats and bat flies was also determined on the cave level (Table 2). Overall, sites Laguna Perdida,

Mastatal, Barra Honda, Emus, Corredores and Tunel ICE 2 demonstrated the highest levels of *Bartonella* prevalence, although Laguna Perdida, Corredores, Barra Honda and Tunel ICE 2 had relatively small sample sizes.

The PCR-positive blood samples came from 27 individual bats from 11 of the 15 independent cave sites (73.3%). PCR-positive blood samples were isolated from six species: *Carollia perspicillata* (70.3%; 19/27) from Laguna Perdida (1 individual), Emus (5), Tunel ICE 2 (2), El Duende (2), Tunel Arenal (2), Mastatal (5), mine El Aguacate (1) and Venado (1); one *Desmodus rotundus* from Laguna Perdida (3.7%; 1/27); *Pteronotus gymonotus* (11.1%; 3/27) from Barra Honda (1) and Corredores (2), one *Lonchophylla robusta* from Locos por el Bosque (3.7%, 1/27), *Macrophyllum macrophyllum* from El Duende (11.1% 3/27), and *Pteronotus mesoamericanus* from El Duende (1) and Tunel Arenal (2).

Bats infected with *Bartonella* demonstrated diversity in characteristics such as their diet and abundance in the cave roosts sampled. *Bartonella* was detected in six species of bats, of which four bat species are newly described to have *Bartonella* infection: *Lonchophylla robusta, Macrophyllum macrophyllum, Pteronotus gymonotus* and *Pteronotus mesoamericanus*. Other bats harboring *Bartonella* in this study such as *Carollia perspicillata* and *Desmodus rotundus* have previously been described as hosts for *Bartonella* species (Bai *et al.* 2011, Judson *et al.* 2015, Becker *et al.* 2018).

The PCR-positive ectoparasites were organized into host-pairs to determine whether the host was also positive (Table 3). The ectoparasite species that were newly described to be infected with *Bartonella* in Costa Rica include: *Strebla vespertilionis, Trichobius pallidus, Megistopoda aranea, Trichobius sparsus, Trichobius johnsonae,*

Strebla galindoi, Trichobius uniformis, Trichobius dunni, Trichobius lionycterdis, and *Strebla hertigi,* although some *Trichobius* bat flies in Judson et al.'s 2015 paper were only identified to the genus level and could be *T. pallidus, T. sparsus, T. johnsonae, T. lionycterdis* or *T. uniformis.* Of the 20 PCR-positive bat flies, only 13 of their bat hosts had blood samples to test for *Bartonella.* Principally, Table 2 shows that only three out of the 13 of the PCR-tested bat hosts for positive bat flies were also positive for *Bartonella*. All three of these bat hosts came from Cave Mastatal and all three were *Carollia perspicillata,* although each parasitized by a different *Trichobius* spp.: *Trichobius uniformis, Trichobius pallidus* and *Trichobius dunni.* The results of this table further support the claim that bat flies are more commonly infected with *Bartonella* than their bat hosts. It also suggests that *Trichobius* bat flies on *Carollia perspicillata* species in Mastatal may be transferring bartonellae to their bat hosts or vice versa.

3.3 Phylogeny of bat and bat Fly *Bartonella* isolates

Of the 27 PCR-positive blood samples, 18 (7%, 18/251) displayed strong enough bands in the confirmation PCR to be sent out for sequencing by Macrogen Inc. Of the 18 amplified *Bartonella* sequences from blood, eight were considered clean enough for phylogenetic analysis (>90% pairwise identity). Of the 18 PCR-positive ectoparasite pools, 13 (20%, 13/64) displayed strong enough bands in the confirmation PCR to be sent out for sequencing by Macrogen Inc. Of the 13 amplified *Bartonella* sequences from ectoparasites, five were considered clean enough for phylogenetic analysis (>90% pairwise identity).

Phylogenetic analysis of the 13 identified *Bartonella* sequences, eight obtained from bats and five from bat flies, as well as strains from bats and bat flies from previous studies in Costa Rica, Belize, Guatemala and Panama, and globally named reference species revealed 11 genetic variants, with four newly described genotypes from this study (Figure 8, Tables 3 & 4). These 11 genetic variants clustered into nine clades of 96.0%-99.2% similarity. The hosts that the isolates were obtained from, as well as the caves those hosts were found are identified in Table 4 for globally identified species and Table 5 for Central American isolates. The clades that the isolates from this study fell into, as well as host and cave information, is provided in Table 6.

B5, an isolate from a *Carollia perspicillata* bat from Emus and B14, an isolate from another *Carollia perspicillata* from Tunel Arenal, clustered into **clade I** with KJ816687 SJ112, a *Bartonella* isolate from a *Paratrichobius longicrus* bat fly collected from *Artibeus lituratus* in Costa Rica in 2015 and HM597200 sp.B29230, an isolate from a *Phyllostomus discolor* bat in Guatemala. These isolates clustered with 96.4-99.2% identity. B5 and B14 displayed 99.2% identity, suggesting they are the same genotype. The creation of this clade suggests similarities among *Bartonella* strains from the same bat species from different caves included in this study (Emus and Tunel Arenal), as well as similarities between this studies' strains and those from Judson *et al.*'s 2015 study in Costa Rica and Bai *et al.*'s 2011 study in Guatemala. It also suggests similarities between strains in different bat species (*Carollia perspicillata* and *Phyllostomus discolor*). Lastly, this clade represents similarities in *Bartonella* spp. in bats and bat flies (*Carollia perspicillata* and *Phyllostomus discolor* bats and a *Paratrichobius longicrus* bat fly).

B11, from a *Carollia perspicillata* bat from El Duende, clustered into **clade II** with KJ816691 SJ117, an isolate from a *Trichobius joblingi* bat fly collected from a *Carollia perspicillata* in Costa Rica in 2015. These two strains demonstrated 99.6% identity, suggesting that they are an identical strain. B11 also clustered in this clade with B10, another isolate from this study, originating from another *Carollia perspicillata* bat from El Duende and with B7 from this study, a *Carollia perspicillata* from Emus. In this clade was also MG799405, an isolate from a *Desmodus rotundus* bat in Belize, as well as with HM597202 sp.B29110, an isolate from a *Glossophaga soricina* in Guatemala, as well as MG799426, and MG799430, both from *Desmodus rotundus* in Belize. Like clade I, clade II suggests similarities in the bartonellae found in the same bat species (*Carollia perspicillata*) from different caves in this study (El Duende and Emus). Moreover, clade II also shows overlap in *Bartonella* strains between bats and bat flies (*Carollia perspicillata, Desmodus rotundus, Glossophaga soricina* bats and *Trichobius joblingi* bat fly). The *Trichobius joblingi* species, which was collected from a *Carollia perspicillata* has also been recorded parasitizing *Glossophaga soricina* and *Desmodus rotundus* bat hosts, which are commonly roosting together (Table 1), suggesting that this bat fly species and related *Bartonella* species could be shared between these bats (Bertola *et al.* 2005, França *et al.* 2013, Dick and Gettinger 2005). *Trichobius joblingi* has been recorded in studies from Brazil as having the highest mean abundance across various bat species, suggesting that this ectoparasite is a generalist in the bat species it parasitizes (França *et al.* 2013; Eriksson *et al.* 2011). This is logical given the diversity of bat species represented in this shared clade. Lastly, this clade demonstrates sharing of strains among bats and bat flies in the countries of Costa Rica, Guatemala and Belize.

B16, a *Bartonella* isolate from a *Carollia perspicillata* bat from Mastatal, only clustered in **clade III** with KJ816686 SJ116, an isolated from a *Trichobius joblingi* bat fly from a *Carollia castanea* from Costa Rica in 2015, with 97.0% identity. This clade suggests a sharing of a strain specific to Costa Rica, as well as potentially a *Bartonella* strain that is specific to *Carollia* spp. and their bat fly parasites. Most importantly, it supports the idea that bats and bat flies are sharing similar *Bartonella* isolates.

B4, B9 and BE9 clustered into their own clade, **clade IV**, with 100% identity to each other. B4 is a *Bartonella* variant from a *Carollia perspicillata* bat at Emus, while B9 is a *Bartonella* variant from a *Carollia perspicillata* at Tunel ICE 2 and BE9 is a variant from a *Trichobius pallidus* bat fly from a *Carollia perspicillata* from Mastatal. The 100% identity of these isolates suggests that three isolates, collected from two different bat individuals of the same species, *Carollia perspicillata*, at two different caves, share the same *Bartonella* isolate as a *Trichobius pallidus* bat fly from a different individual of the same bat species from a third cave. Thus, these three isolates may represent one newly described genotype.

BE2, a *Bartonella* isolate from a *Megistopoda aranea* bat fly from an *Artibeus jamaicensis* bat from Tres Rios, clustered with KJ816677 SJ129 into **clade V**, an isolate from a *Paratrichobius dunni* bat fly collected from a *Uroderma bilobatum* bat from Costa Rica in 2015. These isolates had 99.2% identity, suggesting they may be another shared strain.

BE14, an isolate from a *Trichobius lionycterdis* bat fly from a *Lonchorhina aurita* bat from Los Araya, also did not meet the criteria for a clade with any other sequence. This suggests that BE14 may be a newly described genotype. Thus, BE14 was given its

own clade, **clade VI.** This is possible because *Bartonella* spp. from these species of bat and bat fly have yet to be described in Costa Rica.

BE3, a *Bartonella* variant from a *Trichobius pallidus* bat fly collected from a *Pteronotus mesoamericanus* from Emus did not meet the criteria for a clade with any of the included sequences. Thus, it was given its own clade, **clade VII.** These species' *Bartonella* isolates have not been previously described in Costa Rica.

BE4, a *Bartonella* isolate from a *Trichobius pallidus* bat fly from a *Pteronotus mesoamericanus* bat in Los Araya, clustered with HM597205 sp.B29134, an isolate from a *Pteronotus davyi* from Guatemala clustered into **clade VIII.** The two isolates only clustered with each other, demonstrating 96.4% identity. Clade VIII thus further suggests the presence of similar *Bartonella* spp. in Costa Rican bat flies and Guatemalan bats. Moreover, it supports the claim that *Bartonella* may be associated with different species of bats, since the *Trichobius pallidus* bat fly was found parasitizing a *Pteronotus mesoamericanus* and it clustered with an isolate from a *Pteronotus davyi.*

BE1, an isolate from an *Exastinion clovisi* bat fly from an *Anoura cultrata* did not cluster into a clade with previously identified isolates, with the closest relationship to any previously identified strain being 94.0% identity with KJ816688 SJ102, an isolate that was found in an *Anoura geoffroyi, Artibeus lituratus, Sturnira mordax, Sturnira mordax-Megistopoda proxima* and an *Anoura geoffroyi* in Costa Rica in 2015. This suggests that BE1 may be a newly described genotype. Thus, BE1 was given its own clade, **clade IX.** Interestingly, the most similar strain for this isolate was associated with *Anoura* spp., the genus of the host bat. Markedly, this new genotype was isolated from a bat species that had yet to be sampled, *Anoura cultrata.*

Isolates from this study did not cluster with any globally identified species, suggesting that *Bartonella* spp. in Costa Rican bats and bat flies may represent novel species. Moreover, this suggests that spillover potential to animals and humans may be limited, although past studies have identified and cultured *Bartonella* spp. from bats that were known to cause illness in humans (Lin *et al.* 2010). However, it is important to note that two strains from a previous study in Costa Rica, KJ816689 and KJ816667, clustered with *Bartonella washoensis,* which has caused meningitis in humans and has been found to infect several mammalian species including domesticated animals and wildlife (Kosoy *et al.* 2003; Probert *et al.* 2009). Additionally, in Figure 8, a Costa Rican isolate from the previous study's sampling (KJ816668) was included and clustered with *Bartonella quintana* with 100% identity. However, this isolate was obtained from GenBank and was excluded from the published paper, likely due to potential contamination from these researcher's PCR positive control which was *Bartonella quintana.*

To visualize these phylogenetic trees and corroborate the relationships among strains, stratified trees were produced (Figures 2-7). The trees represent the phylogenetic relationships among strains from this study (Figure 2), strains from this study compared to Costa Rican strains from bats and bat flies collected in 2015 (Figure 3), strains from this study compared to Belizean strains from bats (Figure 4), strains from this study compared to Guatemalan isolates from bats (Figure 5), strains from this study compared to two Panamanian isolates from bat flies (Figure 6), and strains from this study compared to the 22 globally identified *Bartonella* reference species (Figure 7). No significant differences were observed in the phylogenetic relationships among the strains

when stratified. Thus, relationships established by global phylogenetic analysis were further corroborated by these trees.

4. Discussion

Overall, *Bartonella* spp. were found to be diverse, abundant, and potentially widely shared among bats and bat fly species in Costa Rica and Central America, while also displaying a degree of host-specificity and regional structure. My study expanded existing scientific knowledge on the prevalence and diversity of *Bartonella* in Costa Rican bats and bat flies by providing a new dataset, which included bat and bat fly species that had not previously been tested and described as PCR-positive for *Bartonella*. My thesis also contributed to the literature by describing four new *Bartonella* genotypes through phylogenetic analysis. Many of the new genotypes were identified from newly sampled bat and bat fly species (such as an *Exastinion clovisi* bat fly from an *Anoura cultrata*), suggesting that further research, specifically studies sampling more species of bats and bat flies, are needed to fully unveil the level of diversity of bartonellae in Central American bat and bat fly populations.

My thesis also contributed to the field by sampling bats and bat flies from diverse cave roost sites across Costa Rica, representing wet forest, dry forest and agricultural landscape types. Past studies on *Bartonella* in bats and bat flies in Central America did not sample extensively within cave roosts. To my knowledge, these studies primarily sampled bats at non-cave roost sites. The importance of sampling bats directly in their optimal roosts, caves, cannot be understated. Past studies have established that bat species roosting in caves may experience a higher density of ectoparasite infestations (ter

Hofstede and Fenton 2005). Thus, by sampling bats within this specific roost type, my study provided a baseline for the relationship between cave-roosts and variables such as inter-species transmission of *Bartonella* among bats, dynamics and interactions related to cave-specific bat flies and bat host species, as well as potential studies into how anthropogenic disturbance may differentially affect *Bartonella* prevalence in cave roosts.

My results also corroborated previous associations related to the role of bats and bat flies as potential reservoir hosts and arthropod vectors, respectively. Previous findings from Judson *et al.* suggested *Bartonella* spp. are more prevalent in Costa Rican bat flies (52.7%) than Costa Rican bats (33.3%) (Judson *et al.* 2015). Although my study found an overall lower *Bartonella* prevalence in both bats and bat flies, it also found a higher prevalence in bat flies (31.1%) than bat hosts (14.6%). This supports the potential role of bat flies as arthropod vectors for *Bartonella,* which would be expected to have higher rates of infection than their host counterparts if they are acting as vectors for *Bartonella*.

Another major outcome of this study is that identical strains of *Bartonella* were found in Costa Rican bats and bat flies, thus suggesting the potential for sharing of strains. This evidence supports the bat-host bat fly-vector relationship because it asserts that bats and bat flies may be able to both harbor these strains and potentially transmit them to one another. As previously mentioned, the higher prevalence of *Bartonella* in bat flies implicates bat flies as the vector transferring the shared *Bartonella* spp. to bat hosts in this relationship.

However, as suggested by past studies, the sharing of *Bartonella* isolates and high prevalence of *Bartonella* in bat flies only loosely supports their potential role as arthropod vectors. Shared strains found in bat flies and their bat hosts does not prove

their competency as vectors of *Bartonella* (Billeter *et al.* 2012; Judson *et al.* 2015). Although bat and bat flies may share *Bartonella* strains, bat flies may not be efficiently transmitting Bartonella species to their hosts. Rather, bat flies may be acting as incompetent reservoirs for *Bartonella*, where they become infected through a blood-meal from their bat hosts, but do not pass on that *Bartonella* to other bat hosts. Another challenge to this hypothesis is that not all bat flies who were positive for *Bartonella* had hosts who were positive for *Bartonella* (Table 2). This could be explained by vertical transmission of *Bartonella* from bat flies to pupae, leading to higher *Bartonella* prevalence in bat flies than their bat hosts. Morse *et al.* have previously shown that bat flies can transmit *Bartonella* to their pupae via vertical transmission (Morse *et al.* 2012). This could explain why bats harboring positive bat flies may not be positive for *Bartonella* as well. Another potential explanation is that bat flies collected were new arrivals who had not yet taken a blood meal from their new host.

Regardless, my findings are congruent with other studies of bat flies as arthropod vectors for *Bartonella* (Judson *et al.* 2015), as well as other studies that have implicated other arthropod vectors such as ticks, mites and lice (Breitschwerdt *et al.* 2000; Judson *et al.* 2015). In order to fully investigate the relationships between bartonellae, arthropod vectors (including bat flies, mites and ticks) and bats, controlled laboratory experiments must be conducted as well. Moreover, in terms of the transmission dynamics of *Bartonella* in bats, the prevalence of *Bartonella* in tick and mite populations collected from bats should be determined as well. Determining prevalence in these populations is a potential future direction of this study, as mite and tick samples were also taken from the bats I sampled.

Another notable finding from this study was that identical and highly similar (those that clustered into clades) strains were found in bats species and bat flies from the same species of bat hosts (i.e., *Carollia perspicillata* and *Trichobius pallidus* from a *Carollia perspicillata* in clade IV). This could suggest bat-bat fly host-specificity of certain *Bartonella* genotypes, such as the one genotype represented by B4, B9 and BE9, which would be specific to *Carollia perspicillata* and bat flies that parasitize *C. perspicillata*. However, strains of *Bartonella* from different bat host and bat fly species with different bat host species also clustered together in many of the clades (clades I, II, III, V, VII, VIII). Two of these clades (clades III and VIII) represented strains from the same genus of bat, suggesting some association between *Bartonella* strains from bats and bat flies originating from bats of the same genus. Moreover, some bat species such as the highly abundant and almost always present the roost sites sampled (Table 1), *Carollia perspicillata*, appeared to be infected with a large diversity of strains across multiple clades (clades I, II, III, IV). This could potentially be due to more opportunities to become infected with other *Bartonella* strains by having a wide distribution among cave sites, which each have populations constituted by bat, bat fly and bartonellae species. However, this could also be a result of more overall sampling of this species because of their accessibility as abundant and widely distributed in the roost sites. Thus, more downstream analysis of sequences isolated from this species would be expected compare to other bat species. The same level of diversity of *Bartonella* strains may exist among other bat hosts and bat flies originating from other bat hosts as well. More extensive sampling would be needed to investigate these dynamics. Overall, it seems that some *Bartonella* genotypes were more “generalist” in the bat species and bat fly species they

infected (i.e., BE2), whereas other genotypes were more specific (i.e., B4 or B9). Further studies might be conducted to investigate the host specificity of *Bartonella* strains on bat and bat fly species levels.

General patterns in geographic and spatial dynamics as they relate to *Bartonella* spp. in Costa Rican bats and bat flies were revealed in phylogenetic analyses as well. My phylogenetic tree revealed overlap between strains from 2018 Costa Rican bats and Guatemalan bats and bat flies. Additionally, overlap was observed between 2018 Costa Rican bat sequences from this study and sequences from common Vampire bats in Belize. Isolates from bats and bat flies from this study also fell into multiple clades with isolates obtained from bats and bat flies in Costa Rica in 2015. Bat fly isolates from Costa Rica in 2015 also clustered with bat sequences from Belize. Overall, these associations suggest a wide geographic distribution of *Bartonella* spp. in bats, as well as potential sharing of these *Bartonella* between different bat populations in different countries. Additionally, within this study, *Bartonella* strains from bat and bat fly species from different caves from distant regions of the country also clustered together. One example of these general patterns is clade I, in which B5, an isolate from a *Carollia perspicillata* bat from Emus and B14, an isolate from another *Carollia perspicillata* from Tunel Arenal, clustered with KJ816687 SJ112, a *Bartonella* isolate from a *Paratrichobius longicrus* bat fly collected from *Artibeus lituratus* in Costa Rica in 2015 and HM597200 sp.B29230, an isolate from a *Phyllostomus discolor* bat in Guatemala. Notably, these four isolates from different geographic locations clustered with 96.4-99.2% identity. Additionally, B5 and B14 are presumed to be the same genotype. This is notable because this clade suggests that within Costa Rica strains are being shared

between bats in entirely different parts of the country. Emus Cave, where B5 originated from, is in the southwest of the country, whereas Tunel Arenal, where B14 originated from, is in the North (Figure 1). Additionally, the strain from Costa Rican in 2015 originated from Coto Brus, which is also in the south, but is distinctly separate from Emus Cave. Within caves, there was also a high level of diversity of *Bartonella* strains. For example, Los Araya had *Bartonella* isolates in different clades such as BE4 and BE14. In this case, these isolates came from different hosts, a *Trichobius pallidus* bat fly from a *Pteronotus mesoamericanus* bat and a *Trichobius lionycterdis* bat fly from a *Lonchorhina aurita* bat, respectively. In other cases, such as B4 and B7, isolates from the same cave and the same bat hosts also clustered into different clades. This suggests more than one *Bartonella* strain circulating throughout these cave bat and bat fly populations.

A comprehensive example of patterns of *Bartonella* species in bats and bat flies in various caves from this study is represented in clade IV, where B4, B9 and BE9 clustered with 100% identity to each other. B4 is a *Bartonella* variant from a *Carollia perspicillata* bat at Emus, while B9 is a *Bartonella* variant from a *Carollia perspicillata* at Tunel ICE 2 and BE9 is a variant from a *Trichobius pallidus* bat fly from a *Carollia perspicillata* from Mastatal. The 100% identity of these isolates suggests that three isolates, collected from two different bat individuals of the same species, *Carollia perspicillata*, at two different caves, share the same *Bartonella* isolate as a *Trichobius pallidus* bat fly from a different individual of the same bat species from a third cave. This interestingly suggests the sharing of a 100% identical strain of *Bartonella* between bats and bat flies at three entirely different cave sites (Emus, Tunel ICE 2 and Mastatal).

Ultimately, although most isolates from countries included in this study consistently fell together into the same clades (clade I, clade II, clade IV), similar *Bartonella* spp. were found among different bat and bat fly host species from different countries and locations throughout Costa Rica. This suggests a general geographic pattern of *Bartonella* spp. from Costa Rica clustering together due to high levels of similarity, but also emphasizes the diversity and wide geographic distribution of these wild bartonellae.

In assessing spillover risk to humans and domestic animals, it was found that *Bartonella* isolates from bats and bat flies in this study did not cluster with *Bartonella* species known to cause infection in humans and other animals and did not have significant overlap with any globally identified species (Figure 7). Interestingly, Judson *et al.* 2015 found two Costa Rican bat fly *Bartonella* variants genetically like *Bartonella washoensis,* a strain commonly found in rodent species, as well as their flies that is known to cause endocartitis in humans (Kosoy *et al.* 2003). In order to fully assess potential for *Bartonella* spillover from bat and bat fly species to other animals and humans, further analysis should be conducted with more globally identified species, as well as potentially uncultured species such as *Candidatus Bartonella mayotimonensis*, which is known to cause endocartitis in humans and has been found in bats in North America (Lin *et al.* 2010; Lilley *et al.* 2017). Markedly, positive PCR products were not cultured in this study. Since past studies have suggested that bats and bat flies are often co-infected with multiple strains of *Bartonella* and single-locus PCR is limited in its ability to characterize full genotypes, another future direction of this study would be to culture the blood samples from bats and bat flies and conduct multi-locus PCR assays (Bai *et al.* 2011). Not culturing bacteria in this study may have resulted in an

underrepresentation of the number of strains being shared by hosts and their parasites, as well as overall underrepresentation of the true level of diversity of *Bartonella* species in bats and bat flies.

Ultimately, this study was comprehensive in comparing *Bartonella s*trains from bats and bat flies to the most well-characterized and commonly known globally named *Bartonella* species, but the analysis could be expanded in further investigations to include candidatus bacteria, which bat species likely currently harbor, evidenced by the novelty of studies on *Bartonella* in bats and bat flies as well as the high level of diversity of bartonellae demonstrated by these studies. Additional *Bartonella* species such as: *Bartonella talpae, B. rattimassiliensis, B. ancashensis, B. florencae, B. phoceensis,* and *B. schoenbuchensis* could be included as well.

5. Conclusions and Recommendations

Overall, I found that *Bartonella* strains are prevalent and diverse in Costa Rican cave-dwelling bats and bat flies. My study expanded on existing knowledge related to this subject, as well as provided additional data on the potential host (bat) – vector (bat fly) relationship. Moreover, this study added further information related to the host and geographic patterns of *Bartonella* strains in bat and bat fly species in Costa Rica in particular, and Central America more generally. This study also contributed four new genotypes of *Bartonella* to the field. These genotypes were isolated primarily from bat and bat fly species that were not previously sampled, suggesting need for future research to continue to identify novel *Bartonella* strains in a range of bat and bat fly species. Thus, future investigations into the strains that Neotropical bats and bat flies share, as well as

further studies on the vector potential of bat flies, are needed to clarify the role of bats and bat flies in potential *Bartonella* spillover to humans.

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7. Tables

Table 1. Bat species sampled (for all sample types) at each site.

Cave Name	Bat species sampled
Laguna Perdida	*Carollia perspicillata* (2/5; 40%), *Anoura cultrata* (2/5; 40%), *Desmodus rotundus* (1/5; 20%)
Emus	*Carollia perspicillata* (7/9; 78%), *Pteronotus mesoamericanus* (2/9; 22%)
Corredores	*Pteronotus gymnonotus* (6/6, 100%)
Tunel ICE 2	*Artibeus jamaicensis* (2/10; 20%), *Pteronotus* mesoamericanus (3/10; 30%), *Carollia perspicillata* (4/10; 40%), *Saccopteryx bilineata* (1/10; 10%)
Damas	*Carollia perspicillata* (1/2; 50%), *Desmodus rotundus* (1/2; 50%)
Mastatal	*Carollia perspicillata* (10/10; 100%)
Tunel Arenal	*Carollia perspicillata* (12/41; 29.3%), *Desmodus rotundus* (6/41; 14.6%)
El Duende	*Glossophaga soricina* (10/41; 24.4%), *Pteronotus mesoamericanus* (13/41; 31.7%) *Carollia perspicillata* (13/42; 31%), *Desmodus rotundus* (1/42; 2.4%), *Glossophaga commisarisi* (12/42; 29%), *Glossophaga soricina* (1/42; 2.4%), *Macrophyllum macrophyllum* (2/42; 4.8%), *Pteronotus mesoamericanus* (13/42; 31%)
Los Araya	*Carollia perspicillata* (18/53; 34%), *Diphylla ecaudata* (1/53; 1.9%), *Lonchophylla robusta* (10/53; 18.9%), *Lonchorhina aurita* (8/53; 15.1%), *Pteronotus mesoamericanus* (9/53; 17%), *Tonatia saurophila* (1/53; 1.9%), *Trachop cirrhosus* (6/53; 11.3%)
Locos por el Bosque	*Lonchophylla robusta* (10/10, 100%)
El Peñon/La Grande	*Carollia perspicillata* (3/21, 14.2%), *Balantinopteryx plicata* (5/21, 24%), *Desmodus rotundus* (9/21, 43%), *Phyllostomus hastatus* (4/21, 19.0%)
Venado*	*Carollia perspicillata* (12/48, 25%), *Lonchophylla robusta* (6/48, 12.5%), *Lonchorhina aurita* (6/48, 12.5%), *Pteronotus gymonotus* (7/48, 14.6%), *Pteronotus mesoamericanus* (16/48, 33.3%),

	Pteropteryx kappleri (1/48, 2.1%)
Barra Honda*	*Pteronotus gymnonotus* (2/2; 100%)
Minas del Aguacate	*Carollia perspicillata* (11/33, 33.3%), *Desmodus rotundus* (8/33, 24.2%), *Phyllostomus hastatus* (5/33, 15.2%), *Pteronotus mesoamericanus* (7/33, 21.2%), *Trachops cirrhosus* (2/33 6.1%)

*Venado cave is represented as Gabinarraca and Barra Honda is represented as Pozo Hendiondo in Figure 1.

**Tres Rios is excluded, as it is not a cave roost.

Table 2. Total *Bartonella*-PCR prevalence (bat and bat flies) by cave sites sampled across Costa Rica in 2018.

Cave Name	Prevalence	PCR-Positive	Total Sampled
Laguna Perdida	80%	4	5
Emus	39%	7	18
Corredores	33%	2	6
Tunel ICE 2	33%	3	9
Damas	29%	2	7
Mastatal	52%	11	21
Tunel Arenal	10%	4	42
El Duende	8%	4	48
Los Araya	9%	5	58
Locos por el Bosque	10%	1	10
El Peñon/La Grande	0%	0	20
Venado*	2%	1	44
Barra Honda*	50%	1	2
Minas del Aguacate	4%	1	27
Tres Rios	25%	1	4
Total		47	321

*Venado cave is represented as Gabinarraca and Barra Honda is represented as Pozo Hendiondo in Figure 1.

Table 3. *Bartonella*-PCR prevalence in bat hosts of *Bartonella*-PCR positive bat flies sampled from caves across Costa Rica in 2018.

Cave	Bat host	Positive (+/-)	Bat fly (# of individuals)	Positive (+/-)
Damas	*Carollia perspicillata**	N/A	*Trichobius pallidus (3)*	+
Damas	*Desmodus rotundus**	N/A	*Strebla vespertilionis (8)*	+
Laguna Perdida	*Anoura cultrata**	N/A	*Exastinion clovisi (2)*	+
Laguna Perdida	*Anoura cultrata**	N/A	*Exastinion clovisi (1)*	+
Tres Rios	*Artibeus jamaicensis**	N/A	*Megistopoda aranea (2)*	+
Emus	*Pteronotus mesoamericanus*	-	*Trichobius pallidus (3)*	+
Emus	*Carollia perspicillata*	+	*Trichobius pallidus (1)*	+
Tunel ICE 2	*Pteronotus mesoamericanus**	N/A	*Trichobius sparsus (1)*	+
Tunel Arenal	*Carollia perspicillata*	-	*Strebla vespertilionis (1)*	+
Los Araya	*Pteronotus mesoamericanus*	-	*Trichobius johnsonae (2)*	+
Los Araya	*Pteronotus mesoamericanus*	-	*Trichobius pallidus (2)*	+
Los Araya	*Pteronotus mesoamericanus*	-	*Trichobius sparsus (1)*	+
Los Araya	*Tonatia saurophila*	-	*Trichobius pallidus (2)*	+
Los Araya	*Lonchorhina aurita*	-	*Strebla galindoi (1)*	+
Mastatal	*Carollia perspicillata*	+	*Trichobius uniformis (2)*	+
Mastatal	*Carollia perspicillata*	+	*Trichobius pallidus (1)*	+
Mastatal	*Carollia perspicillata*	+	*Trichobius dunni (2)*	+
Mastatal	*Carollia perspicillata*	-	*Trichobius pallidus (2)*	+
Mastatal	*Carollia perspicillata*	-	*Strebla hertigi (2)*	+
Mastatal	*Carollia perspicillata**	N/A	*Trichobius pallidus (2)*	+

*No blood sample taken

Table 4. Globally identified *Bartonella* spp. with *gltA* isolates used as reference sequences for phylogenetic analysis and their accession numbers.

GenBank Accession Number	*Bartonella* species	Host type	Host species for specific strain	Location
AF470616	*Bartonella washoensis*	Rodents, Rabbits	*Spermophilus beecheyi* (squirrel)	United States
AF204273	*Bartonella alstatica*	Rodents, Rabbits	*Oryctolagus cuniculus* (rabbit)	France
AF204272	*Bartonella birtlesii*	Rodents, Rabbits	*Apodemus sp.* (mouse)	Costa Rica
AY584852	*Bartonella taylorii*	Rodents, Rabbits	*Clethrionomys rufocanus* (vole); *Apodemus peninsulae* (mouse); *Microtus fortis* (vole)	Costa Rica
DQ683195	*Bartonella rochalimae*	Humans	*Homo sapiens*	Peru
MH019303	*Bartonella clarridgeiae*	Domestic animals	Domestic cat	Brazil
KT327031	*Bartonella tribocorum*	Rodents, Rabbits	*Apodemus uralensis* (mouse)	Georgia
GU056192	*Bartonella elizabethae*	Human	N/A*	N/A
EU111798	*Bartonella queenslandensis*	Rodents, Rabbits	Australian rat	Australia
AY724769	*Bartonella melophagi*	Ectoparasites (Non-bat fly)	Sheep ked	N/A
AJ278183	*Bartonella schoenbuchii*	Cervids, cattle, ruminants	Deer	Germany
AY254308	*Bartonella chomelii*	Cervids, cattle, ruminants	*Bos taurus*	France
AF207827	*Bartonella doshiae*	Rodents, rabbits	Woodland rodent	United Kingdom
AF293392	*Bartonella capreoli*	Cervids, cattle, ruminants	Roe deer	France

KJ909848	*Bartonella bovis*	Cervids, cattle, ruminants	*Bos taurus* (dairy cattle)	Israel
CP014012	*Bartonella bacilliformis*	Humans	*Homo sapiens*	United States
EF605279	*Bartonella tamiae*	Humans	*Homo sapiens*	Thailand
LS483373	*Bartonella quintana*	Humans	*Homo sapiens*	
MH019304	*Bartonella henselae*	Domestic animals	Domestic cat	Brazil
KY913638	*Bartonella koehlerae*	Ectoparasites (Non-bat fly)	Flea from domestic cat	Chile
EU111793	*Bartonella rattaustraliani*	Rodents, rabbits	Australian rat	Australia
EU111803	*Bartonella coopersplainsesis*	Rodents, rabbits	Australian rat	Australia
CP034103	*Brucella melitensis*	Humans	*Homo sapiens*	China

*Host information for sequence not available in GenBank. Represented in phylogenetic trees as most commonly associated host.

Table 5. Unidentified *Bartonella* spp. from Central American *gltA* isolates used as reference sequences for phylogenetic analysis and their accession numbers.

GenBank Accession Number	*Bartonella* species	Host type	Host species (bat-bat fly)*	Location	Associated study (if published)
KJ816666 (SJ101)	N/A	Bat	*Anoura geoffroyi*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816688 (SJ102)	N/A	Bat	*Anoura geoffroyi*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816680 (SJ103)	N/A	Bat-bat fly pairs	*Artibeus lituratus, Sturnira mordax, Sturnira mordax-Megistopoda proxima, Anoura geoffroyi-Anastrebla modestini, Sturnira lilium*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ16672 (SJ104)	N/A	Bat	*Vampyressa thyone*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816683 (SJ105)	N/A	Bat	*Carollia castanea*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816675 (SJ106)	N/A	Bat	*Artibeus lituratus*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816684 (SJ107)	N/A	Bat	*Platyrrhinus vittatus*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816678 (SJ108)	N/A	Bat fly	*Glossophaga soricina-Trichobius dugesii*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816682 (SJ109)	N/A	Bat fly	*Artibeus jamaicensis-Aspidoptera phyllostomasis*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816685 (SJ111)	N/A	Bat fly	*Artibeus lituratus-Aspidoptera delatorrei*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816687 (SJ112)	N/A	Bat fly	*Artibeus lituratus-Paratrichobius longicrus*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816690 (SJ114)	N/A	Bat-bat fly pairs	*Carollia sowelli, Carollia sowelli–Strebla guajiro*	Coto Brus, Costa Rica	Judson *et al.* 2015

KJ816686 (SJ112)	N/A	Bat fly	*Carollia castanea*– *Trichobius joblingi*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816691 (SJ117)	N/A	Bat fly	*Carollia perspicillata*- *Trichobius joblingi*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816665 (SJ118)	N/A	Bat	*Phyllostomus discolor*- *Trichobius costalimai*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816681 (SJ119)	N/A	Bat fly	*Micronycteris microtus*– *Trichobius keenani*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816671 (SJ121)	N/A	Bat fly	*Sturnira lilium*– *Aspidoptera delatorrei*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816673 (SJ122)	N/A	Bat fly	*Sturnira lilium*– *Aspidoptera delatorrei*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816676 (SJ124)	N/A	Bat	*Myotis keaysi*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816669 (SJ125)	N/A	Bat fly	*Myotis keaysi*– *Anatrichobius scorzai*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816667 (SJ126)	N/A	Bat fly	*Myotis keaysi*– *Anatrichobius scorzai*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816679 (SJ127)	N/A	Bat fly	*Sturnira lilium*– *Aspidoptera delatorrei*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816692 (SJ128)	N/A	Bat fly	*Uroderma bilobatum*- *Paratrichobius dunni*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816677 (SJ129)	N/A	Bat fly	*Uroderma bilobatum*- *Paratrichobius dunni*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816674 (SJ130)	N/A	Bat fly	*Sturnira lilium*- *Aspidoptera delatorrei*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816670 (SJ131)	N/A	Bat fly	*Sturnira lilium*– *Megistopoda proxima*	Coto Brus, Costa Rica	Judson *et al.* 2015
KJ816689 (SJ132)	N/A	Bat fly	*Myotis keaysi*– *Basilia* sp.	Coto Brus, Costa Rica	Judson *et al.* 2015

HM597187	sp. B29042	Bat	*Desmondus rotundus*	Guatemala	Bai *et al.* 2011
HM597188	sp. B29043	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597189	sp. B29044	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597190	sp. B29107	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597191	sp. B29108	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597192	sp. B29114	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597193	sp. B29102	Bat	*Pteronotus davyi*	Guatemala	Bai *et al.* 2011
HM597194	sp. B29109	Bat	*Pteronotus davyi*	Guatemala	Bai *et al.* 2011
HM597195	sp. B29119	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597196	sp. B29122	Bat	*Desmodus rotundus*	Guatemala	Bai *et al.* 2011
HM597197	sp. B29111	Bat	*Artibeus totecus*	Guatemala	Bai *et al.* 2011
HM597198	sp. B29116	Bat	*Phyllostomus discolor*	Guatemala	Bai *et al.* 2011
HM597199	sp. B29126	Bat	*Carollia perspicillata*	Guatemala	Bai *et al.* 2011
HM597200	sp. B29230	Bat	*Phyllostomus discolor*	Guatemala	Bai *et al.* 2011
HM597201	sp. B29115	Bat	*Phyllostomus discolor*	Guatemala	Bai *et al.* 2011
HM597202	sp. B29110	Bat	*Glossophaga soricina*	Guatemala	Bai *et al.* 2011
HM597203	sp. B29105	Bat	*Pteronotus davyi*	Guatemala	Bai *et al.* 2011
HM597204	sp. B29112	Bat	*Phyllostomus discolor*	Guatemala	Bai *et al.* 2011
HM597205	sp. B29134	Bat	*Pteronotus davyi*	Guatemala	Bai *et al.* 2011
HM597206	sp. B29137	Bat	*Sturnira lilium*	Guatemala	Bai *et al.* 2011
HM597207	sp. B29172	Bat	*Micronycteris microtis*	Guatemala	Bai *et al.* 2011
JX416251	N/A	Bat fly	*Strebla diaemi*	Panama	Morse *et al.* 2012
JX416254	N/A	Bat fly	*Paradyschiria lineata*	Panama	Morse *et al.* 2012
MG799404	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018

MG799405	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799419	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799420	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799421	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799422	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799423	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799424	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799425	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799426	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799428	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799429	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018
MG799430	N/A	Bat	*Desmodus rotundus*	Belize	Becker *et al.* 2018

* When the organism sampled was a bat fly, the bat species that the parasite was collected from was indicated. Whether or not the host was positive is indicated as well.

Table 6. *Bartonella* genetic variants, their host-vector pairs, and phylogenetic clades in global phylogenetic analysis.

Code (Identical isolates)	Clade no.	*Bartonella* (bat (+/-) -bat fly)*	Cave Location
B4 (B9, BE9)	IV	*Carollia perspicillata*	Emus
B5 (B14)	I	*Carollia perspicillata*	Emus
B7	II	*Carollia perspicillata*	Emus
B9 (B4, BE9)	IV	*Carollia perspicillata*	Tunel ICE 2
B10	II	*Carollia perspicillata*	El Duende
B11(KJ816691 SJ117)	II	*Carollia perspicillata*	El Duende
B14 (B5)	I	*Carollia perspicillata*	Tunel Arenal
B16	III	*Carollia perspicillata*	Mastatal
BE1***	IX	*Anoura cultrata*** -Exastinion clovisi*	Laguna Perdida
BE2 (KJ816677 SJ129)	V	*Artibeus jamaicensis**-*Megistopoda aranea*	Tres Rios
BE3***	VII	*Pteronotus mesoamericanus (-) – Trichobius pallidus*	Emus
BE4	VIII	*Pteronotus mesoamericanus (-) -Trichobius pallidus*	Los Araya
BE9 (B4, B9)	IV	*Carollia perspicillata (+) - Trichobius pallidus*	Mastatal
BE14***	VI	*Lonchorhina aurita (-) -Trichobius lionycterdis*	Los Araya

*When the organism sampled was a bat fly, the bat species that the parasite was collected from was indicated. Whether or not the host was positive is indicated as well.
**Blood sample was not collected from host.
***This sequence clustered into its own clade by itself.

8. Figures

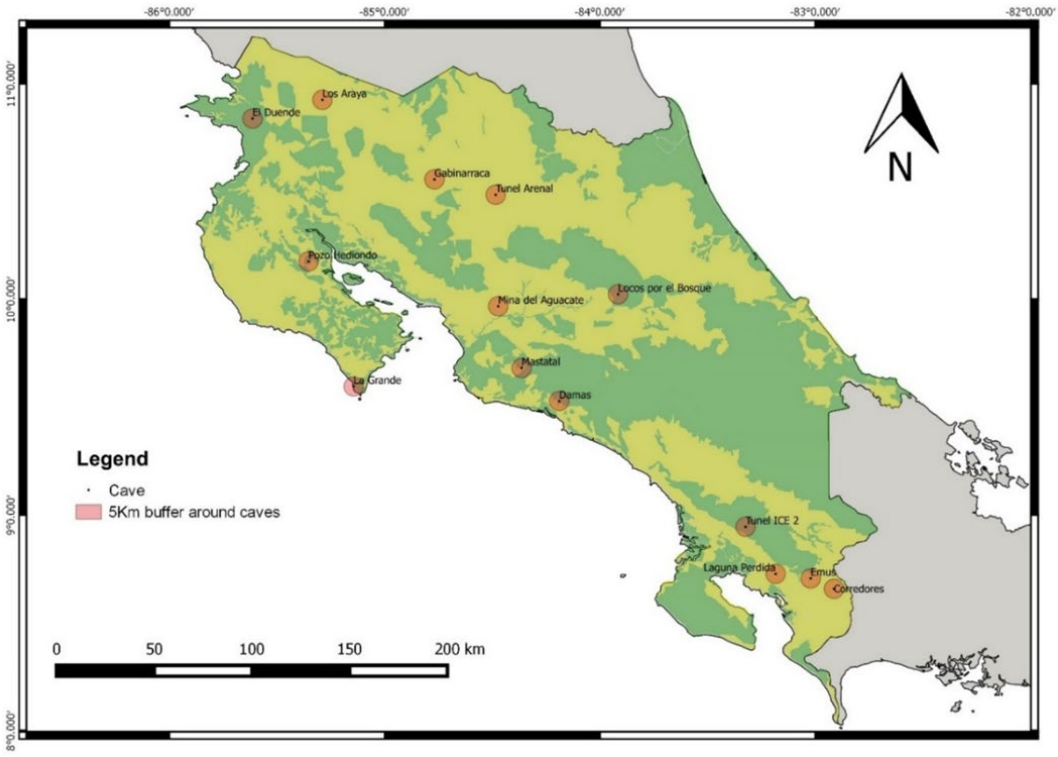

Figure 1. Map of roosts sampled for bats and bat flies in 2018. Venado cave is represented as Gabinarraca and Barra Honda is represented as Pozo Hendiondo.

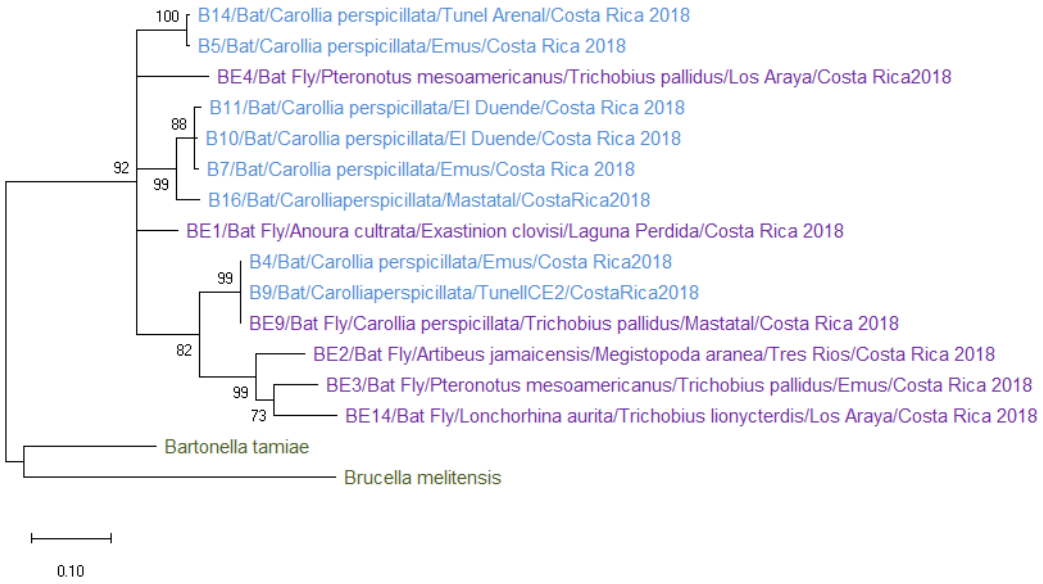

Figure 2. Bayesian phylogenetic tree of *Bartonella gltA* isolates (349 bp) from 2018 Costa Rican bats and bat flies. Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. *Bartonella tamiae*, strain Th329 and *Brucella melitensis*, colored in **green**, were used as an outgroup, in accordance with other publications (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015).

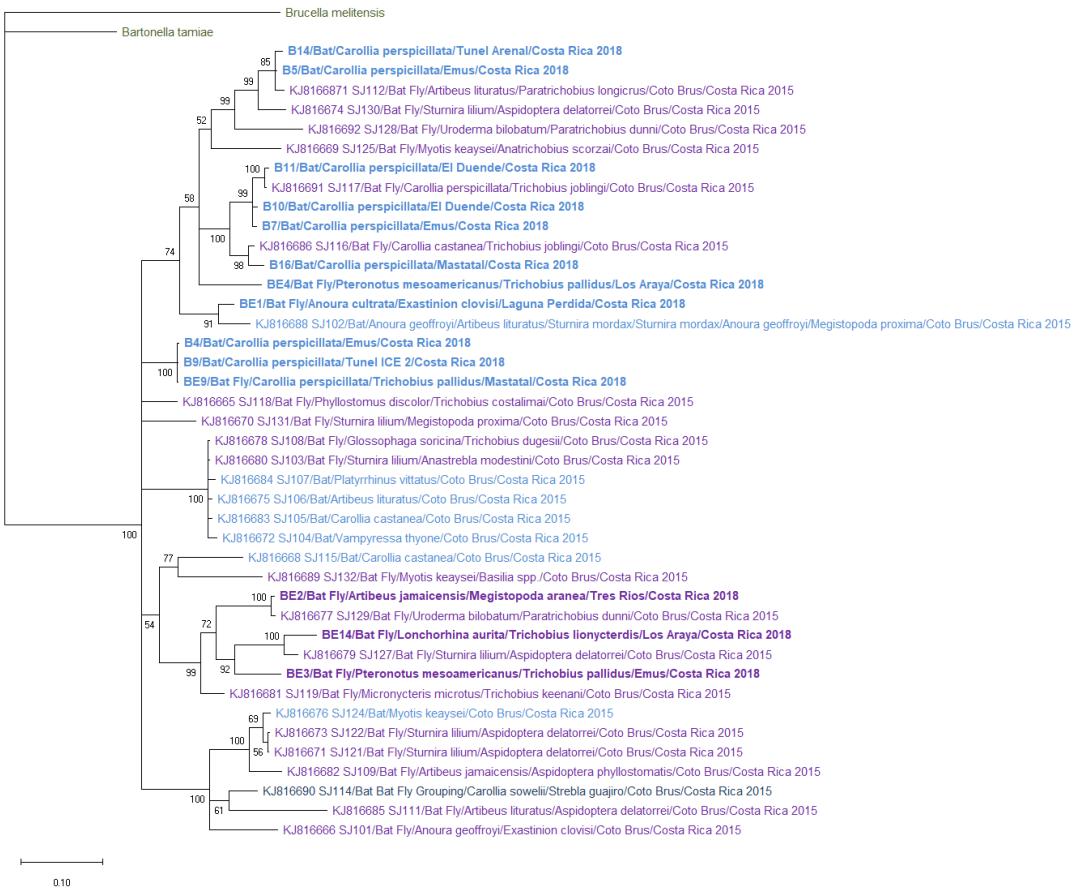

Figure 3. Bayesian phylogenetic tree of *Bartonella gltA* isolates (269 bp) from 2018 Costa Rican bats and bat flies and 2015 isolates from Costa Rican bats and bat flies (Judson *et al.* 2015). Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. Isolates from this study are highlighted in **bold**. *Brucella melitensis* and *Bartonella tamiae*, strain Th329, colored in **green**, were used as outgroups, in accordance with other publications (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015).

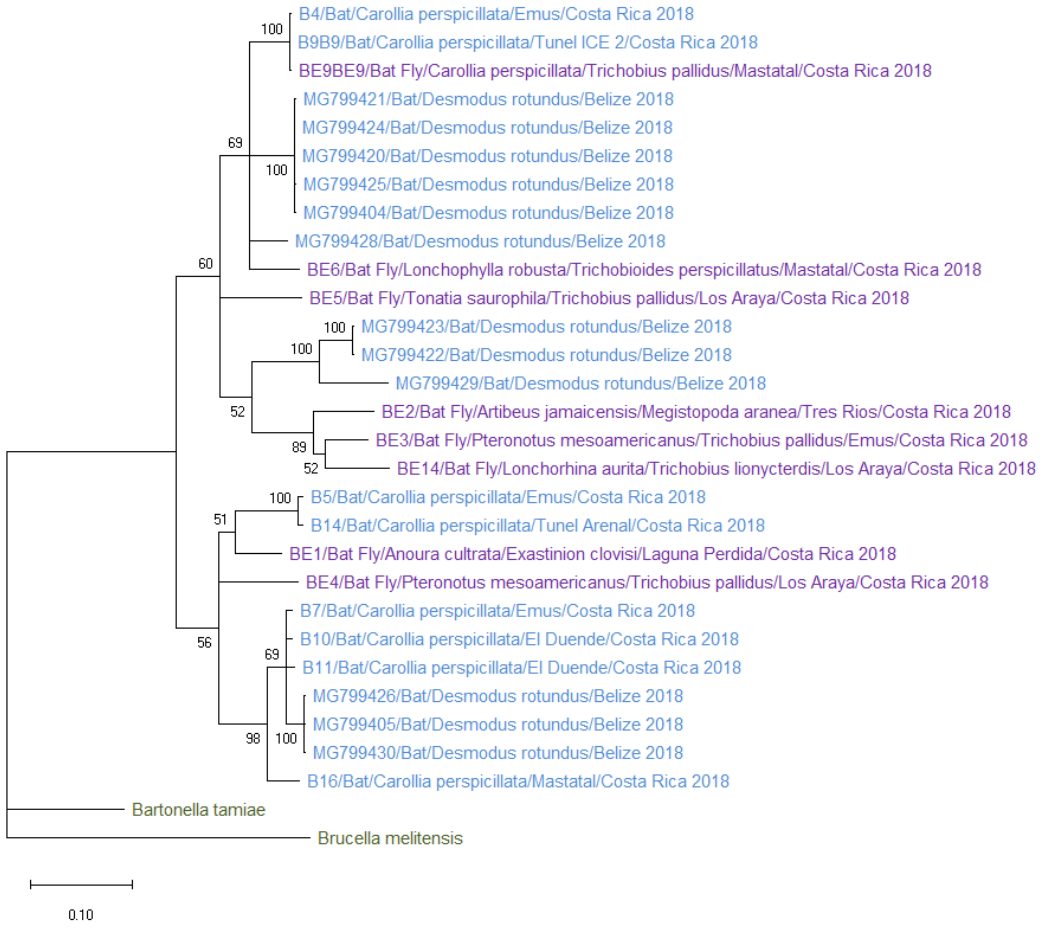

Figure 4. Bayesian phylogenetic tree of *Bartonella gltA* isolates (327 bp) from 2018 Costa Rican bats and bat flies compared to isolates collected in Belize in 2018 (Becker *et al.* 2018). Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. The *Bartonella tamiae*, strain Th329 and *Brucella melitensis*, colored in **green**, were used as an outgroup, in accordance with other publications (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015).

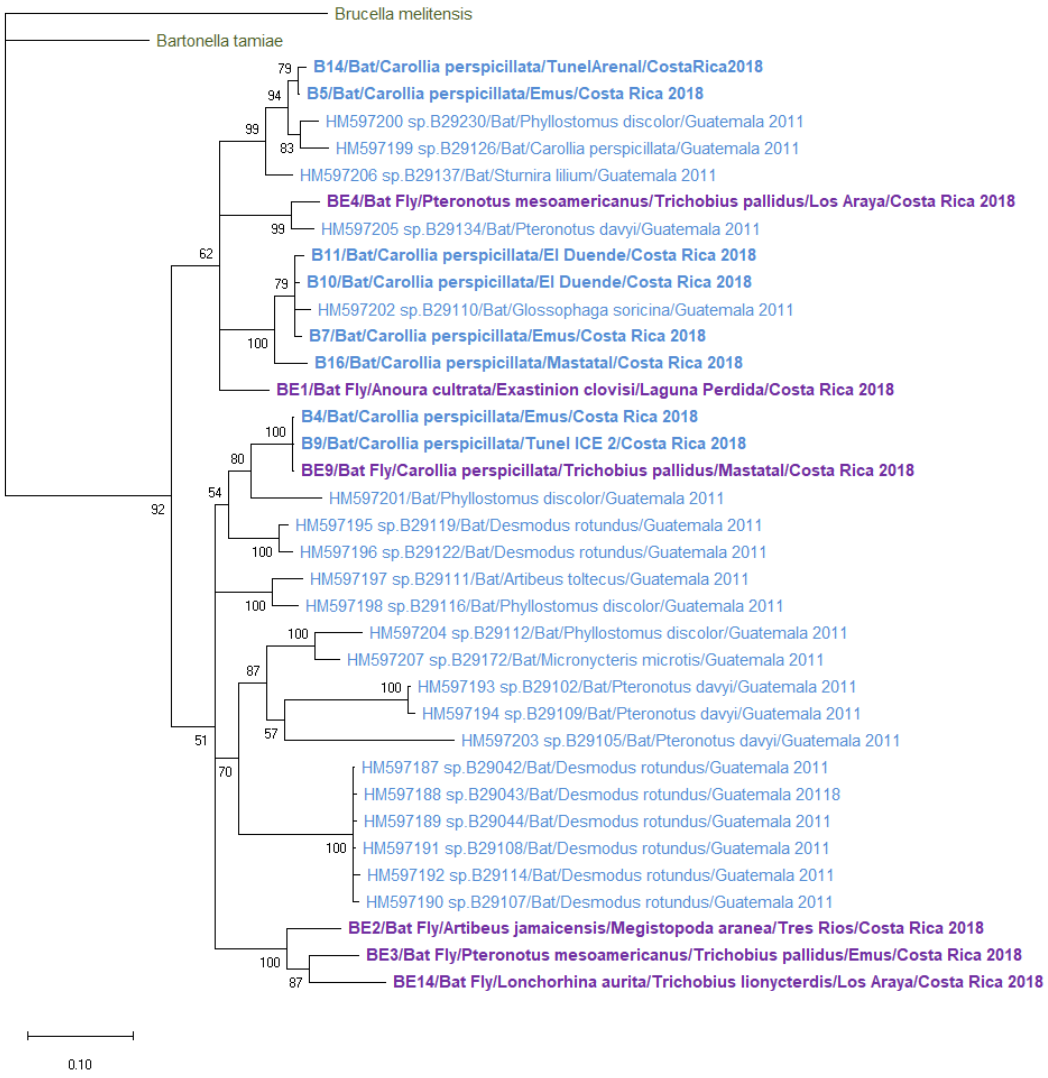

Figure 5. Bayesian phylogenetic tree of *Bartonella gltA* isolates (327 bp) from 2018 Costa Rican bats and bat flies compared to isolates collected in Guatemala in 2011 (Bai *et al.* 2012). Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. Isolates from this study are highlighted in **bold**. *Bartonella tamiae*, strain Th329 and Brucella melitensis, colored in **green**, were used as an outgroup, in accordance with other publications (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015).

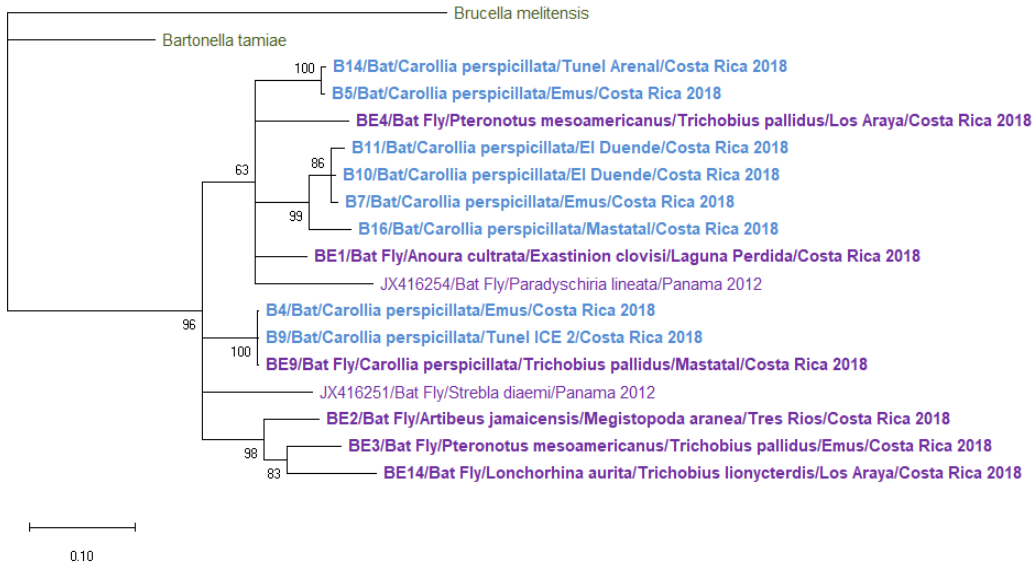

Figure 6. Bayesian phylogenetic tree of *Bartonella gltA* isolates (339 bp) from 2018 Costa Rican bats and bat flies compared to isolates collected in Panama in 2012 (Bai *et al.* 2012). Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. Isolates from this study are highlighted in **bold**. *Bartonella tamiae*, strain Th329 and *Brucella melitensis*, colored in **green**, were used as an outgroup, in accordance with other publications (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015).

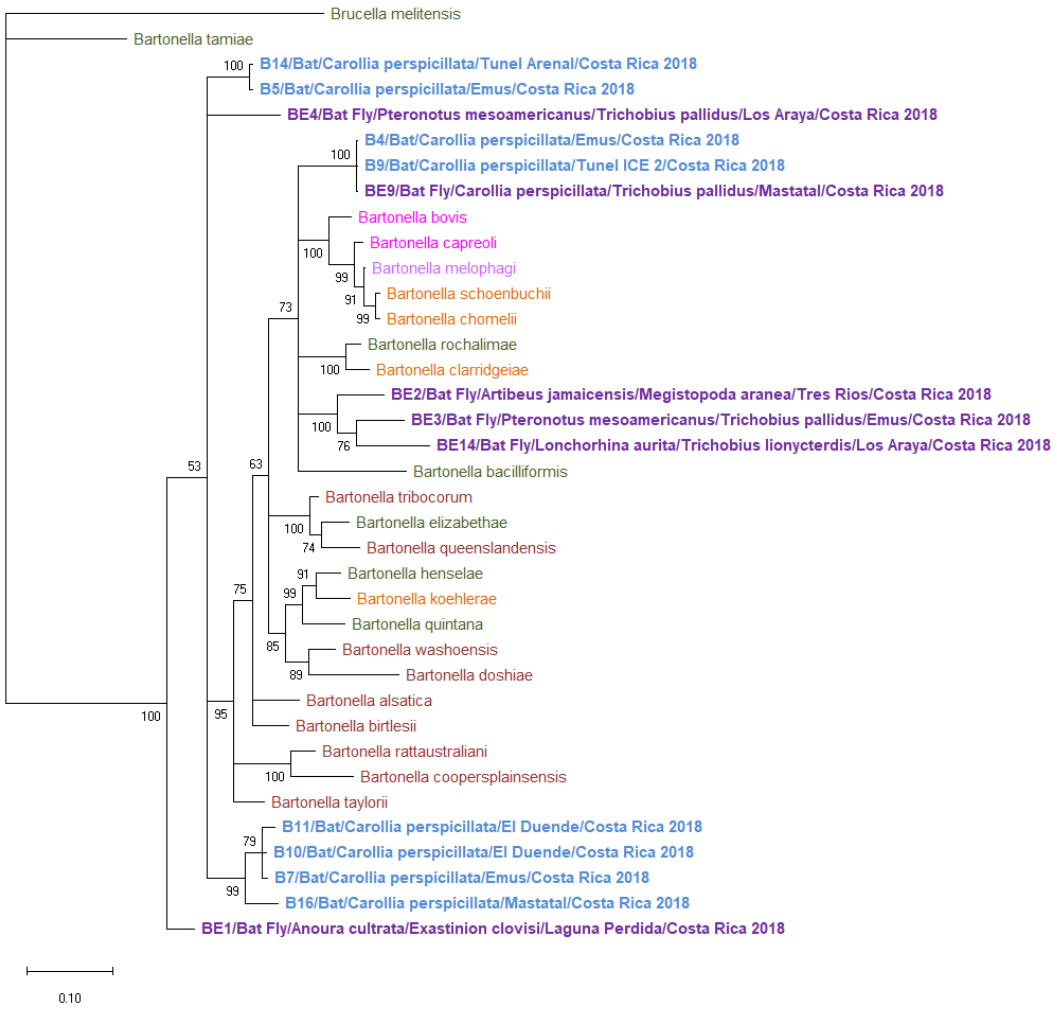

Figure 7. Bayesian phylogenetic tree of *Bartonella gltA* isolates (302 bp) from Costa Rican bats and bat flies and 21 globally named *Bartonella* species. (Kosoy *et al.* 2012; Morse *et al.* 2012; Judson *et al.* 2015). Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. Isolates from this study are highlighted in **bold**. Isolates from rodent and rabbit hosts are colored in **red**, isolates from domesticated animals (cats and dogs) are colored in **orange**, isolates from humans are colored in **green**, isolates from cervids and cattle are in **pink**, and isolates from non-bat fly ectoparasites (ticks, mites) are in **lilac**.

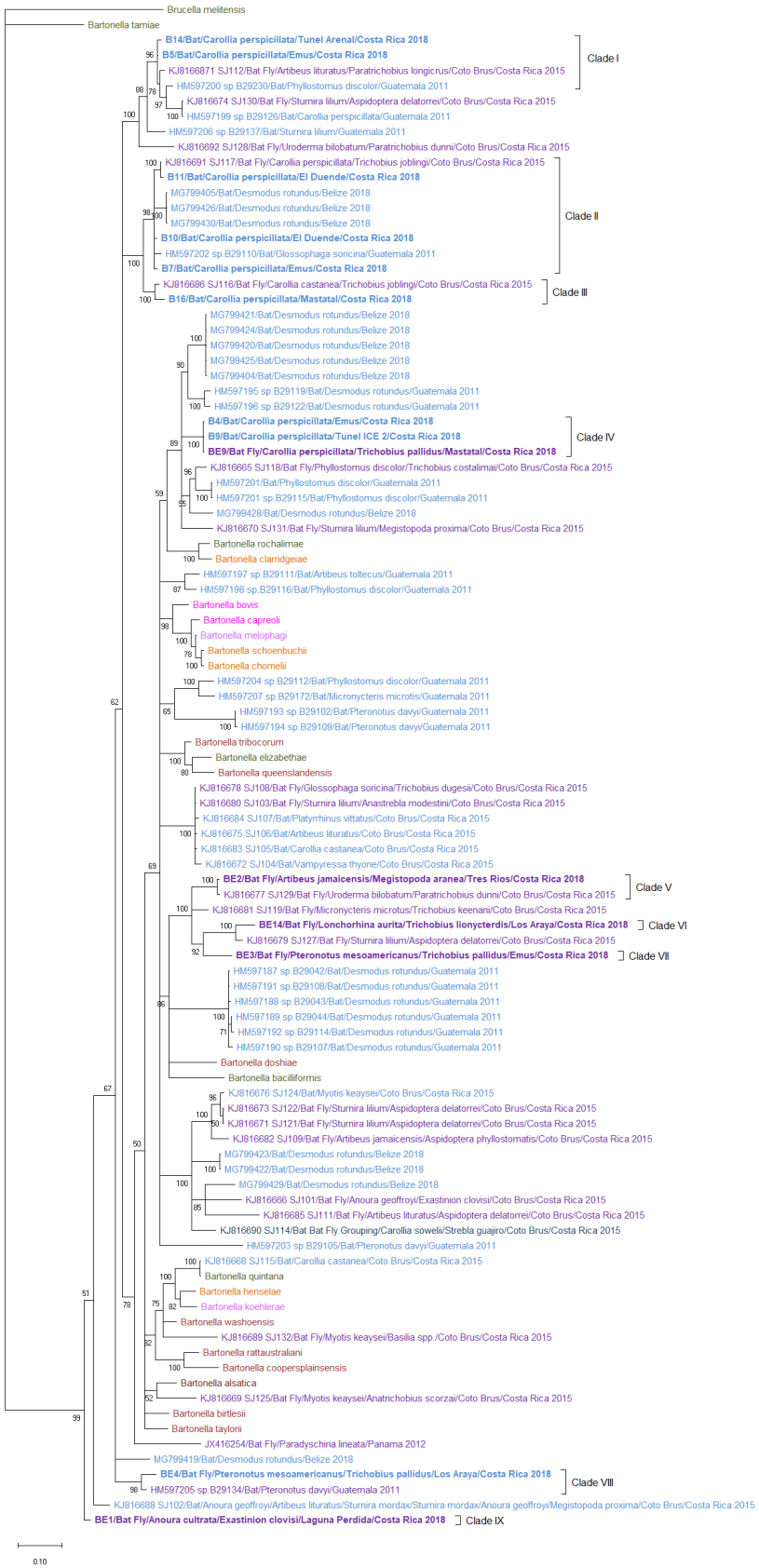
Brucella melitensis
Bartonella tamiae
B14/Bat/Carollia perspicillata/Tunel Arenal/Costa Rica 2018
B5/Bat/Carollia perspicillata/Emus/Costa Rica 2018
KJ816671 SJ112/Bat Fly/Artibeus lituratus/Paratrichobius longicrus/Coto Brus/Costa Rica 2015
HM597200 sp.B29230/Bat/Phyllostomus discolor/Guatemala 2011
KJ816674 SJ130/Bat Fly/Sturnira lilium/Aspidoptera delatorrei/Coto Brus/Costa Rica 2015
HM597199 sp.B29126/Bat/Carollia perspicillata/Guatemala 2011
HM597206 sp.B29137/Bat/Sturnira lilium/Guatemala 2011
KJ816692 SJ128/Bat Fly/Uroderma bilobatum/Paratrichobius dunni/Coto Brus/Costa Rica 2015
KJ816691 SJ117/Bat Fly/Carollia perspicillata/Trichobius joblingi/Coto Brus/Costa Rica 2015
B11/Bat/Carollia perspicillata/El Duende/Costa Rica 2018
MG799405/Bat/Desmodus rotundus/Belize 2018
MG799426/Bat/Desmodus rotundus/Belize 2018
MG799430/Bat/Desmodus rotundus/Belize 2018
B10/Bat/Carollia perspicillata/El Duende/Costa Rica 2018
HM597202 sp.B29110/Bat/Glossophaga soricina/Guatemala 2011
B7/Bat/Carollia perspicillata/Emus/Costa Rica 2018
KJ816686 SJ116/Bat Fly/Carollia castanea/Trichobius joblingi/Coto Brus/Costa Rica 2015
B16/Bat/Carollia perspicillata/Mastatal/Costa Rica 2018
MG799421/Bat/Desmodus rotundus/Belize 2018
MG799424/Bat/Desmodus rotundus/Belize 2018
MG799420/Bat/Desmodus rotundus/Belize 2018
MG799425/Bat/Desmodus rotundus/Belize 2018
MG799404/Bat/Desmodus rotundus/Belize 2018
HM597195 sp.B29119/Bat/Desmodus rotundus/Guatemala 2011
HM597196 sp.B29122/Bat/Desmodus rotundus/Guatemala 2011
B4/Bat/Carollia perspicillata/Emus/Costa Rica 2018
B9/Bat/Carollia perspicillata/Tunel ICE 2/Costa Rica 2018
BE9/Bat Fly/Carollia perspicillata/Trichobius pallidus/Mastatal/Costa Rica 2018
KJ816665 SJ118/Bat Fly/Phyllostomus discolor/Trichobius costalimai/Coto Brus/Costa Rica 2015
HM597201/Bat/Phyllostomus discolor/Guatemala 2011
HM597201 sp.B29115/Bat/Phyllostomus discolor/Guatemala 2011
MG799428/Bat/Desmodus rotundus/Belize 2018
KJ816670 SJ131/Bat Fly/Sturnira lilium/Megistopoda proxima/Coto Brus/Costa Rica 2015
Bartonella rochalimae
Bartonella clarridgeiae
HM597197 sp.B29111/Bat/Artibeus toltecus/Guatemala 2011
HM597198 sp.B29116/Bat/Phyllostomus discolor/Guatemala 2011
Bartonella bovis
Bartonella capreoli
Bartonella melophagi
Bartonella schoenbuchii
Bartonella chomelii
HM597204 sp.B29112/Bat/Phyllostomus discolor/Guatemala 2011
HM597207 sp.B29172/Bat/Micronycteris microtis/Guatemala 2011
HM597193 sp.B29102/Bat/Pteronotus davyi/Guatemala 2011
HM597194 sp.B29109/Bat/Pteronotus davyi/Guatemala 2011
Bartonella tribocorum
Bartonella elizabethae
Bartonella queenslandensis
KJ816678 SJ108/Bat Fly/Glossophaga soricina/Trichobius dugesii/Coto Brus/Costa Rica 2015
KJ816680 SJ103/Bat Fly/Sturnira lilium/Anastrebla modestini/Coto Brus/Costa Rica 2015
KJ816684 SJ107/Bat/Platyrrhinus vittatus/Coto Brus/Costa Rica 2015
KJ816675 SJ106/Bat/Artibeus lituratus/Coto Brus/Costa Rica 2015
KJ816683 SJ105/Bat/Carollia castanea/Coto Brus/Costa Rica 2015
KJ816672 SJ104/Bat/Vampyressa thyone/Coto Brus/Costa Rica 2015
BE2/Bat Fly/Artibeus jamaicensis/Megistopoda aranea/Tres Rios/Costa Rica 2018
KJ816677 SJ129/Bat Fly/Uroderma bilobatum/Paratrichobius dunni/Coto Brus/Costa Rica 2015
KJ816681 SJ119/Bat Fly/Micronycteris microtus/Trichobius keenani/Coto Brus/Costa Rica 2015
BE14/Bat Fly/Lonchorhina aurita/Trichobius lionycterdis/Los Araya/Costa Rica 2018
KJ816679 SJ127/Bat Fly/Sturnira lilium/Aspidoptera delatorrei/Coto Brus/Costa Rica 2015
BE3/Bat Fly/Pteronotus mesoamericanus/Trichobius pallidus/Emus/Costa Rica 2018
HM597187 sp.B29042/Bat/Desmodus rotundus/Guatemala 2011
HM597191 sp.B29108/Bat/Desmodus rotundus/Guatemala 2011
HM597188 sp.B29043/Bat/Desmodus rotundus/Guatemala 2011
HM597189 sp.B29044/Bat/Desmodus rotundus/Guatemala 2011
HM597192 sp.B29114/Bat/Desmodus rotundus/Guatemala 2011
HM597190 sp.B29107/Bat/Desmodus rotundus/Guatemala 2011
Bartonella doshiae
Bartonella bacilliformis
KJ816676 SJ124/Bat/Myotis keaysei/Coto Brus/Costa Rica 2015
KJ816673 SJ122/Bat Fly/Sturnira lilium/Aspidoptera delatorrei/Coto Brus/Costa Rica 2015
KJ816671 SJ121/Bat Fly/Sturnira lilium/Aspidoptera delatorrei/Coto Brus/Costa Rica 2015
KJ816682 SJ109/Bat Fly/Artibeus jamaicensis/Aspidoptera phyllostomatis/Coto Brus/Costa Rica 2015
MG799423/Bat/Desmodus rotundus/Belize 2018
MG799422/Bat/Desmodus rotundus/Belize 2018
MG799429/Bat/Desmodus rotundus/Belize 2018
KJ816666 SJ101/Bat Fly/Anoura geoffroyi/Exastinion clovisi/Coto Brus/Costa Rica 2015
KJ816685 SJ111/Bat Fly/Artibeus lituratus/Aspidoptera delatorrei/Coto Brus/Costa Rica 2015
KJ816690 SJ114/Bat Bat Fly Grouping/Carollia sowelii/Strebla guajiro/Coto Brus/Costa Rica 2015
HM597203 sp.B29105/Bat/Pteronotus davyi/Guatemala 2011
KJ816668 SJ115/Bat/Carollia castanea/Coto Brus/Costa Rica 2015
Bartonella quintana
Bartonella henselae
Bartonella koehlerae
Bartonella washoensis
KJ816689 SJ132/Bat Fly/Myotis keaysei/Basilia spp./Coto Brus/Costa Rica 2015
Bartonella rattaustraliani
Bartonella coopersplainsensis
Bartonella alsatica
KJ816669 SJ125/Bat Fly/Myotis keaysei/Anatrichobius scorzai/Coto Brus/Costa Rica 2015
Bartonella birtlesii
Bartonella taylorii
JX416254/Bat Fly/Paradyschiria lineata/Panama 2012
MG799419/Bat/Desmodus rotundus/Belize 2018
BE4/Bat Fly/Pteronotus mesoamericanus/Trichobius pallidus/Los Araya/Costa Rica 2018
HM597205 sp.B29134/Bat/Pteronotus davyi/Guatemala 2011
KJ816688 SJ102/Bat/Anoura geoffroyi/Artibeus lituratus/Sturnira mordax/Sturnira mordax/Anoura geoffroyi/Megistopoda proxima/Coto Brus/Costa Rica 2015
BE1/Bat Fly/Anoura cultrata/Exastinion clovisi/Laguna Perdida/Costa Rica 2018
Clade I
Clade II
Clade III
Clade IV
Clade V
Clade VI
Clade VII
Clade VIII
Clade IX
0.10

Figure 8. Bayesian phylogenetic tree of *Bartonella gltA* isolates (250 bp) from bats and bat flies from Guatemala, Belize, Panama and Costa Rica, as well as 21 globally identified species. Isolates obtained from bat flies are colored in **purple** and isolates from bats are colored in **blue**. Isolates from this study are highlighted in **bold**. Isolates from rodent and rabbit hosts are colored in **red**, isolates from domesticated animals (cats and dogs) are colored in **orange**, isolates from humans are colored in **green**, isolates from cervids and cattles are in **pink**, and isolates from non-bat fly ectoparasites (ticks, mites) are in **lilac**.