Ecological and evolutionary drivers of hemoplasma infection and bacterial genotype sharing in a Neotropical bat community

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26 specificity

27

Abstract

Most emerging pathogens can infect multiple species, underscoring the importance of understanding the ecological and evolutionary factors that allow some hosts to harbor greater infection prevalence and share pathogens with other species. Investigating such factors can inform surveillance efforts and help forecast disease emergence. However, our understanding of pathogen jumps is primarily based around viruses, despite bacteria accounting for the greatest proportion of zoonoses. Because bacterial pathogens in bats (Order: Chiroptera) can have conservation and human health consequences, studies that examine the ecological and evolutionary drivers of bacterial prevalence and barriers to pathogen sharing are crucially needed. We here studied hemotropic *Mycoplasma* spp. (i.e., hemoplasmas) across a species-rich bat community in Belize over two years. Across 469 bats spanning 33 species, half of individuals and two-thirds of species were hemoplasma positive. Infection prevalence was higher for males, heavier species, and those with larger colony sizes. Hemoplasmas displayed high genetic diversity (21 novel genotypes) and strong host specificity. Evolutionary patterns supported co-divergence of bats and bacterial genotypes alongside phylogenetically constrained host shifts. Bat species centrality to the network of shared hemoplasma genotypes was phylogenetically clustered and unrelated to prevalence, further suggesting rare—but detectable—bacterial sharing between species. Our study highlights the importance of using fine phylogenetic scales when assessing host specificity and suggests phylogenetic similarity may play a key role in host shifts for not only viruses but also bacteria. Such work more broadly contributes to increasing efforts to understand cross-species transmission and epidemiological consequences of bacterial pathogens.

Introduction

Most pathogens that cause disease in humans, domestic animals, and wildlife are capable of infecting multiple host species (Woolhouse, Taylor, & Haydon, 2001). However, predicting which hosts maintain pathogens and identifying their role in disease emergence can be challenging, as many hosts can be infected but not play key roles in the reservoir community (Fenton, Streicker, Petchey, & Pedersen, 2015; Viana et al., 2014). Pathogen jumps between species depends on infection prevalence in the donor host, transmission opportunities between donor and recipient species, and suitability of the recipient host for pathogen replication (Parrish et al., 2008; Plowright et al., 2017). Each of these steps can be shaped by ecological and evolutionary factors (Nishiura, Hoye, Klaassen, Bauer, & Heesterbeek, 2009; VanderWaal & Ezenwa, 2016). For example, small-bodied species can have greater competence, the ability to transmit new infections, than larger species (Downs, Schoenle, Han, Harrison, & Martin, 2019), and host switching is commonly constrained by phylogeny, owing to similarity in immunological barriers to pathogen replication between related species (Streicker et al., 2010). Understanding the ecological and evolutionary factors that allow some species to harbor greater prevalence and have facilitated pathogen sharing could thus guide wildlife surveillance and forecast emergence (Fountain-Jones et al., 2018). Further, examining associations between hosts and pathogens can uncover factors favoring host shifts versus evolutionary codivergence, which is a key requisite for generating such predictions (Geoghegan, Duchêne, & Holmes, 2017; Lei & Olival, 2014).

Given the public health and agricultural burdens of many zoonotic pathogens such as avian influenza virus and rabies virus, most investigations of the determinants of pathogen prevalence and emergence focus on viruses (Geoghegan et al., 2017; Luis et al., 2015; Olival et al., 2017). However, more zoonoses are caused by bacteria than other pathogen taxa (Han,

Kramer, & Drake, 2016), and bacterial pathogens can negatively impact newly infected host species (e.g., *Mycoplasma galliscepticum*, a poultry pathogen, caused rapid population declines in wild house finches; Hochachka & Dhondt, 2000). More attention to bacteria and their propensity for host specificity versus generalism is accordingly important for understanding whether factors that govern cross-species transmission of viruses can be extended to other pathogens (Bonneaud, Weinert, & Kuijper, 2019). Bacterial pathogens have been especially understudied for bats (Lei & Olival, 2014; Mühldorfer, 2013), in contrast to intensive studies of zoonotic viruses (Drexler et al., 2012; Luis et al., 2015). Yet many bacterial pathogens are likely important to bat conservation and human health due to pathogenic effects on bats themselves and their zoonotic potential (Becker et al., 2018; Evans, Bown, Timofte, Simpson, & Birtles, 2009).

To determine the ecological and evolutionary drivers of bacterial prevalence and barriers to pathogen sharing, we focused on hemotropic *Mycoplasma* spp. (i.e., hemoplasmas) in a species-rich bat community in Belize (Fenton et al., 2001; Herrera, Duncan, Clare, Fenton, & Simmons, 2018). The Neotropics have remarkable bat diversity owing to adaptive radiation in the Phyllostomidae (Gunnell & Simmons, 2012), producing a range of feeding strategies (e.g., frugivory, carnivory, sanguivory), body sizes, and roosting preferences (Monteiro & Nogueira, 2011; Wetterer, Rockman, & Simmons, 2000). Hemoplasmas are intracellular erythrocytic bacteria transmitted by direct contact (Cohen et al., 2018; Museux et al., 2009) and also possibly arthropod vectors (Willi, Boretti, Meli, et al., 2007). Hemoplasmas can cause acute and chronic anemia, especially for immunocompromised hosts; however, many animals develop inapparent infections and are asymptomatic (Messick, 2004). As *Mycoplasma* spp. lack many of the metabolic pathways associated with energy production and synthesis of cell components found in other bacteria, they are fully dependent on host cells (Citti & Blanchard, 2013). Hemoplasmas

have therefore been described as mostly host specialists (Pitcher & Nicholas, 2005), yet interspecies and potentially zoonotic transmission can occur (Maggi et al., 2013; Willi, Boretti, Tasker, et al., 2007). Hemoplasmas are common and genetically diverse in bats (Ikeda et al., 2017; Millán et al., 2019; Volokhov et al., 2017), which offers an ideal system for identifying the ecological and evolutionary factors structuring bacterial infection within and between species.

Many cross-species comparisons of pathogen infection risks and sharing have used less-diverse host communities (Johnson et al., 2012; VanderWaal, Atwill, Isbell, & McCowan, 2014) or global datasets of host–pathogen associations that can be limited by heterogeneous sampling effort and variation in pathogen detection methods (Dallas et al., 2019; Huang, Bininda-Emonds, Stephens, Gittleman, & Altizer, 2014). Our focus on a widespread pathogens group in a highly diverse host community allowed us to capitalize on strong host trait variation while controlling for sampling effort and diagnostics methods (Becker, Crowley, Washburne, & Plowright, 2019; Han, Kramer, et al., 2016). Past work has also used host–pathogen networks to characterize contemporary or historic transmission at often coarse taxonomic scales (e.g., pathogen species complexes or genera; Blyton, Banks, Peakall, Lindenmayer, & Gordon, 2014; VanderWaal et al., 2014). However, because bat species can be infected by multiple hemoplasma genotypes, and because genotypes with ≥99% sequence identity of their 16S rRNA genes can represent different species (Volokhov, Hwang, Chizhikov, Danaceau, & Gottdenker, 2017; Volokhov, Simonyan, Davidson, & Chizhikov, 2012), focusing on pathogen genotypes provides finer-scale resolution in identifying the ecological and evolutionary features of species that facilitate pathogen sharing and to identify likely maintenance hosts of bacterial infections (Fountain-Jones et al., 2018).

We asked three questions about the relative contribution of ecological traits and evolutionary history to structuring infection patterns and pathogen sharing. First, what are the

individual and ecological predictors of hemoplasma infection in bats? Second, does the distribution of hemoplasma genotypes across the bat community map onto the bat phylogeny, as might be predicted by host–pathogen codivergence? Lastly, if genotype sharing between host species occurs, which host clades or traits best predict network centrality? We predicted that ecological covariates such as ectoparasitism and large host colonies could increase bacteria risk through vector-borne or density-dependent transmission (McCallum, Barlow, & Hone, 2001; Willi, Boretti, Meli, et al., 2007). We also expected hemoplasma genotypes would be specific to particular host species but that more closely related bats would share hemoplasma genotypes, indicating phylogenetically restricted host shifts (Pitcher & Nicholas, 2005). However, host traits that increase the risk of bacteria exposure, such as occupying a greater diversity of roosts, could also explain pathogen genotype sharing among less closely related hosts (McKee et al., 2019).

Methods

Bat capture and sampling

From April 24 to May 6 2017 and from April 23 to May 5 2018, we sampled 469 bats from 33 species captured in two adjacent areas in the Orange Walk District of Belize: Lamanai Archaeological Reserve (LAR) and Ka'Kabish (KK; Fig. 1). The LAR is bordered by the New River Lagoon, forest, and agriculture, while KK is a remnant patch of forest surrounded by agriculture located 10 km away. At least 44 of the 70 bat species in Belize have been recorded in this region (Herrera et al., 2018; Reid, 1997). Bats were captured with mist nets at the exits of roosts or along flight paths from 19:00 until 22:00. Harp traps were also set from 18:00 to 05:00.

Bats were placed in cloth bags until processing and were identified to species (and sex) based on morphology (Reid, 1997). Reproductive activity was indicated by the presence of

scrotal testes in males and by the evidence of pregnancy or lactation in females. We also visually screened bats for the presence of ectoparasites (i.e., bat flies, ticks, bat bugs, mites; Ter Hofstede, Fenton, & Whitaker, 2004). We collected 3–30 μL of blood (volumes were dependent on bat mass) by lancing the propatagial vein with a sterile needle. Blood was collected with heparinized capillary tubes and stored on Whatman FTA cards to preserve bacterial DNA. Field procedures followed guidelines for safe and humane handling of bats published by of the American Society of Mammalogists (Sikes, Care, & Mammalogists, 2016) and were approved by the Institutional Animal Care and Use Committees of the University of Georgia (A2014 04-016-Y3-A5) and American Museum of Natural History (AMNHIACUC-20170403 and AMNHIACUC-20180123). Fieldwork was authorized by the Belize Forest Department under permits WL/2/1/17(16), WL/2/1/17(19), and WL/2/1/18(16). Sample size was similar between years (2017=202, 2018=267) but varied by site (LAR=365, KK=101). More species were sampled for blood at LAR (*n*=33) than KK (*n*=17; Fig. 1), reflecting site differences in species richness (Herrera et al., 2018). We sampled 1–139 individuals per bat (the maximum was the common vampire bat, *Desmodus rotundus*), with a mean of 14 individuals per bat species (Table S1).

DNA extraction, PCR amplification, and amplicon sequencing

Genomic DNA was extracted from blood on FTA cards using QIAamp DNA Investigator Kits (Qiagen). We tested DNA for hemoplasmas using PCR with primers and procedures described in prior analyses (Volokhov et al., 2017). We included blank FTA punches as an extraction control, ultrapure water as a negative control, and *Candidatus* Mycoplasma haemozalophi DNA as a positive control (Volokhov et al., 2011). Amplicons from PCR-positive samples were purified by electrophoresis and extracted with the QIAquick Gel Extraction Kit (Qiagen).

To determine hemoplasma infection status, all 16S rRNA amplicons were directly sequenced by Macrogen (https://www.macrogenusa.com/). Amplicons were sequenced with the same primers used for PCR amplification and then with internal (walking) primers when needed (Volokhov et al., 2017). Negative DNA samples were tested for amplification quality using the universal PCR primers targeting the mammal mitochondrial 16S rRNA gene (Volokhov, Kong, George, Anderson, & Chizhikov, 2008) or the mitochondrial cytochrome *c* oxidase subunit 1 gene (Clare, Lim, Engstrom, Eger, & Hebert, 2007); all hemoplasma-negative DNA samples gave positive signal in the mitochondrial 16S rRNA gene- and/or the COI-specific PCR assays. All amplified sequences were subjected to chimeric sequence analysis using DECIPHER (Wright, Yilmaz, & Noguera, 2012) and UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011). All hemoplasma sequences have been deposited in GenBank under accession numbers MH245119–MH245194 and MK353807–MK353892; four positive samples were identified as *Bartonella* spp. during sequencing and were considered hemoplasma negative in our analyses.

Bat phylogenetic data

We used the *rotl* and *ape* packages in R to extract a bat phylogeny from the Open Tree of Life and to calculate branch lengths with Grafen's method (Michonneau, Brown, & Winter, 2016; Paradis, Claude, & Strimmer, 2004). To assess hemoplasma genotype sharing as a function of host phylogenetic similarity, we derived pairwise phylogenetic distances between the 33 sampled bat species (Fig. S1). Because more evolutionarily distinct host species could display less frequent bacterial genotype sharing owing to ecological and immunological barriers to pathogen exposure and replication (Huang, Drake, Gittleman, & Altizer, 2015), we used the *picante* package and our bat phylogeny to derive evolutionary distinctiveness (Kembel et al., 2010).

187

188 *Host species trait data*

189 We obtained species-level data on host traits relevant to pathogen transmission from previously
190 published sources (Table S2). We obtained fecundity (litter size, litters per year), body mass, and
191 diet from the Amniote Life History and EltonTraits databases (Myhrvold et al., 2015; Wilman et
192 al., 2014). For foraging ecology, which could affect bacterial exposure (e.g., trophic interactions;
193 Kellner et al., 2018), we defined three dietary guilds: frugivory and nectarivory (*n*=11),
194 insectivory (*n*=18), and carnivory (including sanguivory and piscivory, *n*=4; González-Salazar,
195 Martínez-Meyer, & López-Santiago, 2014). We also considered the proportion of plant-based
196 items in diet. We simplified foraging strata into aerial (*n*=14), arboreal (*n*=16, including
197 scansorial), and ground- or aquatic-level foraging (*n*=3). We also expanded prior compilations of
198 wing aspect ratios and roost preferences to serve as proxies for ecological overlap among species
199 (Fenton et al., 2001; Herrera et al., 2018; Reid, 1997). Roost type was simplified to open (e.g.,
200 only foliage; *n*=6) or closed (e.g., hollows, caves; *n*=27), and roost flexibility was simplified to
201 using one (*n*=16) or multiple roost types (*n*=17). We classified maximum colony sizes as small-
202 to-medium (e.g., under 100 individuals; *n*=20) or large (e.g., hundreds to thousands; *n*=13; Reid,
203 1997; Santana, Dial, Eiting, & Alfaro, 2011), as most values were reported in ranges. We did not
204 record pairwise sympatry (e.g., Luis et al., 2015; McKee et al., 2019) given that all species occur
205 in Belize (Fig. S2). Yet because more widely distributed species could have more opportunities
206 for pathogen sharing due to range overlap, we used the *geosphere* package and data from the
207 International Union for Conservation of Nature to derive geographic range size (Baillie, Hilton-
208 Taylor, & Stuart, 2004; Hijmans, Williams, Vennes, & Hijmans, 2019). Missing species-level
209 traits were taken from other databases, primary literature, or closely related species (Table S2).

210

211 *Individual-level analysis of bat infection status*

212 To analyze individual-level data, we used a phylogenetic generalized linear mixed model
213 (GLMM) to test if hemoplasma infection status varied by sex, reproductive status, year, site, and
214 ectoparasitism while accounting for bat phylogenetic relatedness. We also included interactions
215 between sex and reproduction and between site and year. As vampire bats were banded for a
216 mark–recapture study (Volokhov et al., 2017) and some were sampled between and within years
217 (n=14), we randomly selected one of each recapture. After removing recaptures and missing
218 values (remaining n=323), we fit the phylogenetic GLMM using the *brms* package, default
219 priors, and infection status as a Bernoulli-distributed response. We included random effects for
220 bat species and phylogeny, the latter of which used the phylogenetic covariance matrix (Bürkner,
221 2017). We ran four chains for 20,000 iterations with a burn-in period of 10,000, thinned every 10
222 steps, for a total 4,000 samples. We estimated fixed effects (means and 95% highest density
223 intervals [HDI]) from the posterior distributions of each predictor alongside a Bayesian R^2, and
224 we fit an intercept-only GLMM to quantify the variance explained by only the random effects.

225

226 *Species-level analysis of hemoplasma prevalence*

227 We calculated infection prevalence per species, using the *metafor* package to estimate logit-
228 transformed proportions and sampling variances (Viechtbauer, 2010). We used the *nlme* package
229 to estimate phylogenetic signal as Pagel's λ with a weighted phylogenetic generalized least
230 squares (PGLS) model to account for within-species variance (Garamszegi, 2014). We next used
231 a graph-partitioning algorithm, phylogenetic factorization, to flexibly identify clades with
232 significantly different prevalence estimates at various taxonomic depths. We used the *taxize*

package to obtain a bat taxonomy from the National Center for Biotechnology Information (Chamberlain & Szöcs, 2013) and used the *phylofactor* package to partition prevalence as a Bernoulli-distributed response in a GLM (Washburne et al., 2019). We determined the number of significant bat clades using Holm's sequentially rejective test with a 5% family-wise error rate.

To identify species trait correlates of prevalence, we fit 11 PGLS models (weighted by sampling variance) with body mass, annual fecundity (litters per year * pups per litter), dietary guild, quantitative diet, foraging strata, aspect ratio, roost type, roost flexibility, colony size, geographic range size, and evolutionary distinctiveness as predictors. We also fit PGLS models with sample size and with an intercept. We compared models with Akaike information criterion corrected for small sample sizes (AICc) and estimated R^2 (Burnham & Anderson, 2002).

Hemoplasma phylogenetic analyses and genotype assignment

We compared our 16S rRNA sequences to those in GenBank (Volokhov et al., 2017; Volokhov et al., 2011). Briefly, we aligned sequences using Clustal X, and inter- and intra-species similarity values were generated using BioEdit. Genetic distances were calculated with the Kimura two-parameter and Tamura–Nei models, and the bacterial phylogeny was constructed using MEGA 7 with the minimum evolution algorithm (Kumar, Stecher, & Tamura, 2016).

We assigned hemoplasma genotypes to positive bats based on analysis of the 16S rRNA partial gene (860–1000 bp) sequences in GenBank and their clustering on the phylogeny. Genotypes were designated as novel if (*i*) sequences differed from the closest hemoplasma sequences in GenBank by ≥1.5% and/or (*ii*) if sequence similarity was <1.5% but genotype-specific reproducible mutations (at least two per sequence) were observed between hemoplasma sequences from at least two independent bat samples and the nearest GenBank hemoplasma

sequences. These genotype-specific mutations were further used to differentiate closely related hemoplasma genotypes from our sample. We caution that genotype is not synonymous with species, as analysis of the 16S rRNA gene alone is insufficient for accurate species identification of *Mycoplasma* spp. (Volokhov et al., 2012). Future studies using genomics or housekeeping genes may identify independent but closely related hemoplasma species in our genotypes.

To assess if hemoplasma genotype assignments were associated with site and year, we used χ^2 tests with *p* values generated through a Monte Carlo procedure. Prior to our phylogenetic and network analyses of genotype distributions across bat species (see below), we used another χ^2 test to assess the association between hemoplasma genotype identify and bat host identity.

Evolutionary relationships between bats and hemoplasmas

To determine the degree to which bat hemoplasma genotypes display host specificity and to describe their evolutionary relationships with host species, we used our bat and hemoplasma phylogenies to construct a binary association matrix. To test the dependence of the hemoplasma phylogeny upon the bat phylogeny and thus assess evidence of evolutionary codivergence, we applied the Procrustes Approach to Cophylogeny (PACo) using distance matrices and the *paco* package (Hutchinson, Cagua, Balbuena, Stouffer, & Poisot, 2017). We used a jackknife procedure to estimate the degree to which each bat–genotype link supported a hypothesis of phylogenetic congruence; links were supported if their upper 95% confidence interval was below the mean of all squared jackknife residuals (Balbuena, Míguez-Lozano, & Blasco-Costa, 2013).

Hemoplasma genotype sharing among bat species

We used hemoplasma genotype assignments to create a network, with each node representing a bat species and edges representing shared genotypes among bat species pairs. We built an adjacency matrix using the *igraph* package and used the Louvain method to assess the structure of bat–hemoplasma communities within this network (Csardi & Nepusz, 2006). To test whether the distribution of hemoplasma genotypes across our Neotropical bat species is shaped by host phylogeny, we used two GLMs to predict counts of shared genotypes (Poisson errors) and the presence of sharing (binomial errors) by phylogenetic distance between bat species. We assessed statistical significance using a quadratic assignment procedure via the *sna* package (Butts, 2008).

We calculated two metrics of network centrality to quantify different aspects of how important a node (bat species) is to hemoplasma genotype sharing: degree and eigenvector centrality (Bell, Atkinson, & Carlson, 1999). Whereas degree indicates the number of other species with which a host shares bacterial genotypes (i.e., links per node), eigenvector centrality indicates the tendency for a host to share genotypes with species that also share more genotypes (i.e., connectivity). Eigenvector centrality is thus an extension of degree that can identify hubs of parasite sharing (Gómez, Nunn, & Verdú, 2013). Both metrics were moderately correlated across (ρ=0.59), with many non-zero degree species displaying zero eigenvector centrality. To examine spatial and temporal patterns in host centrality, we built separate adjacency networks per each site and year. We fit separate GLMs to ask how hemoplasma sharing centrality was predicted by site, year, and the two-way interaction. Degree was modeled as a Poisson-distributed response, while eigenvector centrality was logit-transformed and used Gaussian errors. We next applied phylogenetic factorization to each metric and weighted the algorithm by the square-root sample size per species (Garamszegi, 2014). We then fit the same PGLS models used in our prevalence analysis to identify the most competitive trait predictors of bat species centrality to hemoplasma

301 sharing. To assess whether network centrality is associated with hemoplasma prevalence, we
302 lastly fit two weighted PGLS models with each centrality metric as a univariate predictor.
303
304 **Results**
305 *Hemoplasma infection status*
306 We detected sequence-confirmed hemoplasma infection in 239 of 469 individuals (51%; 95%
307 CI: 46–55%), with positive individuals in 23 of the 33 sampled bat species (Table S3). Our
308 phylogenetic GLMM explained 24% of the variation in infection status (Fig. 2A). Males had
309 higher odds of infection than females (OR=2.26, 95% HDI: 1.07–4.92; Fig. 2B), and infection
310 also varied by year, but only at one site (KK; Fig. 2C). Infection status was unrelated to
311 ectoparasitism or reproduction. However, an intercept-only GLMM explained 18% of the
312 variance, suggesting that species random effects were more important than the fixed effects
313
314 *Inter-species variation in hemoplasma prevalence*
315 Across bat species, hemoplasma prevalence ranged from 0% to 100% ($\tilde{x}$=0.37). We estimated
316 Pagel's λ in logit-transformed prevalence to be 0.39, indicating moderate phylogenetic signal.
317 Similarly, phylogenetic factorization identified one bat clade with significantly lower prevalence
318 compared to the paraphyletic remainder: the Emballonuridae (12% infected; Fig. 3A). Our trait-
319 based analysis showed that relatively larger species (β=1.48, p=0.01, R^2=0.24) and those with
320 larger colonies (β=0.67, p=0.06, R^2=0.20) had higher hemoplasma prevalence (Fig. 3B; Table 1).
321 Relatively heavier ($\geq$ 20 g) and larger colony bat species included *Desmodus rotundus*, *Molossus*
322 *rufus*, and *Pteronotus mesoamericanus*, for which infection prevalence was greater than 58%.
323

Hemoplasma genotype diversity

Our phylogenetic analysis identified 29 *Mycoplasma* genotypes in the Belize bat community (Table 2), including three identified prior from vampire bats (VBG1–3; Volokhov et al., 2017). All genotypes demonstrated minor levels of sequence variability (99.5–100%; Table 2). Based on comparisons with sequences from GenBank, 21 of these genotypes are novel hemoplasmas, five are closely related to non-hemoplasma mycoplasmas, and many are related to previously identified *Mycoplasma* spp. and hemoplasmas from other bat species (e.g., Millán et al., 2015), bat ticks (e.g., Hornok et al., 2019), primates (e.g., Madden, Moats, London, Matthew, & Sever, 1974), and rodents (e.g., Goto, Yasuda, Hayashimoto, & Ebukuro, 2010; Fig. S3). A more detailed description of these 29 bacterial genotypes is provided within the Online Supplement.

After controlling for multiple comparisons, our 29 bacterial genotypes were associated with site (χ^2=47.11, p<0.01) and year (χ^2=40.40, p<0.01). Genotype composition was more diverse at LAR (Fig. S4), and KK hemoplasmas were dominated by vampire bat genotypes (VBG1–3). Genotype composition was more idiosyncratic by study year. However, these 29 bacterial genotypes were most strongly associated with bat species (χ^2=3532, p<0.01; Fig. S4).

Bat–hemoplasma evolutionary relationships

Although some hemoplasma genotypes were shared between bat species (i.e., VBG1, CS2, PPM, EF1, AH1–2, MYE, PLU, SP; n=9), most showed strong host specificity (n=20; Table 2). Our coevolutionary analysis (PACo) supported strong congruence between bat and hemoplasma phylogenies (m^2_{XY}=33.76, p<0.01; Fig. 4), suggesting that hemoplasma evolution has mostly tracked bat speciation. However, PACo also demonstrated that only 49% of the 41 unique bat–hemoplasma links displayed significant evidence of coevolution (Fig. S5), and these patterns

were almost exclusively found within the Phyllostomidae (with the exception of *Saccopteryx bilineata* and its *Mycoplasma muris*–like bacterial genotype). The other 21 bat–hemoplasma links therefore displayed evidence of phylogenetic incongruence and thus likely host shifts.

Hemoplasma genotype sharing networks

Within our bat–hemoplasma network, genotype sharing was restricted to five host communities, whereas six genotypes were each restricted to a single bat species (Fig. 5A). GLMs showed that both the frequency and presence of genotype sharing declined with phylogenetic distance between bat species (Poisson: $p<0.001$, $R^2=0.08$; binomial: $p<0.001$, $R^2=0.51$; Fig. 5B).

Bat species shared hemoplasma genotypes with zero to five other species (i.e., degree), and most hosts were not central to the network of genotype sharing (i.e., eigenvector centrality of zero). Six bat species had non-zero eigenvector centrality values that ranged from 37% to 100%, indicating that these hosts generally shared more hemoplasma genotypes with other highly connected hosts. Stratifying our hemoplasma genotype network across sites and years showed that centrality measures varied by space but not time (Fig. S7, Table S4). We observed no hemoplasma genotype sharing at KK, likely reflecting lower host diversity (Herrera et al., 2018).

Phylogenetic factorization identified similar bat clades with significantly different centrality compared to the paraphyletic remainder (Fig. 6A–B). For degree, the algorithm only identified *Artibeus lituratus* as being more central ($x\square=5$) than other bats ($x\square=1.14$). However, phylogenetic factorization identified three taxa in the subfamily Stenodermatinae that had significantly elevated eigenvector centrality: the genera *Artibeus* and *Dermanura* ($x\square=0.67$ compared to $x\square=0.02$ in all other bats), the species *Artibeus lituratus* ($x\square=1$ compared to $x\square=0.12$), and the species *Sturnira parvidens* ($x\square=0.37$ compared to $x\square=0.15$). Mirroring these

results, phylogenetic signal was absent for degree (λ=0) but high for eigenvector centrality (λ=0.93).

Trait-based analyses showed that degree centrality was best predicted by diet (Table S5); bat species feeding more heavily on fruit and nectar shared more bacterial genotypes with other species (β=0.004, p<0.001, R^2=0.20; Fig. 6C). Similarly, eigenvector centrality was best predicted by bat colony size and diet (Table S6); highly central species had small colonies (β_{large}=–1.93, p=0.05, R^2=0.13) and fed more on plants (β=0.03, p<0.01, R^2=0.10; Fig. 6D).

As a final analysis, we assessed whether network centrality (i.e., a bat species' role in hemoplasma genotype sharing) predicted contemporary infection prevalence (Fig. S8). However, we found no associations between species-level infection prevalence and centrality as measured by degree (β=–0.13, R^2=0.03, p=0.42) or eigenvector centrality (β=–0.20, R^2<0.01, p=0.79).

Discussion

By examining the prevalence and distribution of a common bacterial pathogen (hemoplasmas) in a diverse bat community, we expanded analysis of the ecological and evolutionary predictors of bat infection and pathogen sharing beyond viruses. Across the bat community, hemoplasma infection risk was generally higher for males but was better predicted by phylogeny, with large-bodied and large-colony bat species showing greater prevalence. Hemoplasmas showed high diversity and mostly strict host associations, with strong congruence between the bat and hemoplasma phylogenies. Although codivergence was supported by our analyses, we also observed hemoplasma genotype sharing and evidence of historical host shifts between closely related bats. Species most central to this hemoplasma sharing network displayed taxonomic clustering and were disproportionately frugivores and nectarivores. Yet these highly central bat

393 species did not also have the highest hemoplasma prevalence, reinforcing mostly infrequent
394 bacterial sharing between species. Our work reveals phylogenetic patterns in hemoplasma
395 infection in a diverse bat community while contributing to broader efforts to understand the host
396 specificity of bacterial pathogens and their cross-species transmission risks in wildlife.

397 Whereas many bacterial pathogens, including hemoplasmas, are common in bats (Bai et
398 al., 2011; Becker et al., 2018; Ikeda et al., 2017; Mascarelli et al., 2014; Millán et al., 2015;
399 Volokhov et al., 2017), the factors that confer high infection probability are poorly understood.
400 In the Belize bat community, the odds of hemoplasma infection were higher in males, mirroring
401 male-biased transmission previously detected in feline and canine systems (Soto et al., 2017;
402 Walker Vergara et al., 2016). Such patterns could stem from males mounting weaker immune
403 responses than females (Kelly, Stoehr, Nunn, Smyth, & Prokop, 2018) or to male defense of
404 multi-female roosts in many Neotropical bat species (Voigt, von Helversen, Michener, & Kunz,
405 2001). Direct transmission of hemoplasmas has been demonstrated in feline and rodent systems
406 (Cohen et al., 2018; Museux et al., 2009) but only inferred in bats from metagenomic studies
407 detecting these bacteria in saliva (Volokhov et al., 2017). We found weak support for the
408 hypothesis that ectoparasites play a role in infection risks (Hornok et al., 2019; Willi, Boretti,
409 Meli, et al., 2007). Male bias in infection further casts doubt on vector-borne transmission, as
410 female bats generally have elevated ectoparasitism (Frank, Mendenhall, Judson, Daily, & Hadly,
411 2016). Although prior work has suggested some bacterial infections to be endemic in bats
412 (Becker et al., 2018; Millán et al., 2015; Volokhov et al., 2017), we also found yearly infection
413 patterns varied by habitat. Prevalence varied little between years in the LAR but was dynamic in
414 the fragmented site (KK). Fragmentation could promote unstable infection patterns by altering
415 susceptibility (e.g., the microbiota; Ingala, Becker, Holm, Kristiansen, & Simmons, 2019) or

community composition (e.g., relative abundance of competent hosts; Keesing, Holt, & Ostfeld, 2006). Longitudinal studies could confirm these patterns and elucidate transmission routes.

Across Neotropical bats, we found phylogeny to be a better predictor of hemoplasma infection risk than individual traits, site, or year. Phylogenetic factorization identified one clade, the Emballonuridae (*Saccopteryx bilineata* and *Rhynchonycteris naso*), with significantly lower prevalence than all other bats in the community. This moderate phylogenetic signal mirrors comparable effects of phylogeny for bat viruses (Guy, Thiagavel, Mideo, & Ratcliffe, 2019; Schmidt John Paul et al., 2019), similarly suggesting potential for innate differences in species susceptibility or pathogen exposure. Trait-based analyses revealed that this taxonomic pattern was driven by heavier species and those with larger colony sizes having greater hemoplasma prevalence. Small-bodied species could have low prevalence due to small blood volumes and low bacterial titers (Volokhov et al., 2017). Alternatively, the positive, saturating relationship between body mass and bacterial prevalence could be driven by allometric patterns in competence (Downs et al., 2019), in contrast to weak or opposite relationships between mass and viral richness across bats (Guy et al., 2019; Han, Schmidt, et al., 2016; Olival et al., 2017). As larger-bodied bat species can also be more abundant in Neotropical habitat fragments (Herrera et al., 2018), these results suggest land conversion could increase the frequency of bat species most capable of maintaining hemoplasma infection. Similarly, positive relationships between colony size and prevalence could support density-dependent transmission of bacteria (McCallum et al., 2001), whereas mixed support has been found for bat viruses (Streicker et al., 2012; Webber, Fletcher, & Willis, 2017). Future work could test how community-wide infection patterns vary across broader habitat gradients and use multiple bacteria to assess the generality of these trends.

Approximately two-thirds of Neotropical bat species were infected by hemoplasmas, for which we observed high genetic diversity consistent with other studies of this bacterial pathogen in bats (Mascarelli et al., 2014; Millán et al., 2015; Volokhov et al., 2017). However, genotypes detected here were mostly novel and only weakly related to hemoplasmas described elsewhere in Latin America (Ikeda et al., 2017; Millán et al., 2019), with the exception of those previously identified from vampire bats in Peru and Belize (Volokhov et al., 2017). When considering the phylogenetic scale of genotypes, most hemoplasmas were host specific. Over half our hemoplasma communities consisted of a single bat–genotype association, matching the degree of host specificity observed more generally for *Mycoplasma* spp. (Citti & Blanchard, 2013; Pitcher & Nicholas, 2005). When we did detect genotype sharing between species, this occurred mostly between closely related hosts (e.g., PPM was detected in *Pteronotus mesoamericanus* and *P. fulvus*), indicating that bat phylogenetic distance decreased the probability of bacterial transfer.

Analyses to characterize species centrality to the hemoplasma genotype sharing network showed that one species (*Artibeus lituratus*) and the subfamily Stenodermatinae played key roles. This clade, and especially the genera *Artibeus* and *Dermanura* (formerly all classified in *Artibeus*), was the only taxon with non-zero connectivity, and this pattern was reflected in fruit- and nectar-based diets and small colonies being the primary predictors of centrality. The strictly frugivorous Stenodermatinae represents a recent divergence in the Phyllostomidae (Botero-Castro et al., 2013), and high centrality of these species may indicate weaker phylogenetic barriers for bacterial transmission between hosts in this clade. Two other analyses reinforced infrequent and conserved hemoplasma sharing between species. First, phylogenetic patterns in prevalence were distinct from those in genotype sharing centrality (e.g., large-colony species had higher prevalence but lower connectivity), and prevalence accordingly did not predict centrality.

Second, we found general congruence between bat and hemoplasma phylogenies. Although this shows codivergence is a strong evolutionary force, congruence can also stem from preferential jumps to closely related hosts (De Vienne et al., 2013). Though we cannot rule out that some host shifts may be artefacts of the limited resolution of both the phylogenies, our evolutionary analyses and genotype sharing results imply that hemoplasma host shifts are possible yet rare.

By sampling a diverse assemblage of bacterial genotypes in an ecologically and evolutionary rich host community, our work has broader implications for our understanding of disease emergence. Many bacterial pathogens are thought to be generalists and relatively unlikely to specialize in a novel host (Pedersen, Altizer, Poss, Cunningham, & Nunn, 2005; Woolhouse & Gowtage-Sequeria, 2005), in contrast to many viruses in which host shifts are more common owing to high mutation rates and short infectious periods (Geoghegan et al., 2017; Longdon, Brockhurst, Russell, Welch, & Jiggins, 2014). Recent theoretical work suggests host shift speciation may be less common for bacteria because of higher phenotypic plasticity (e.g., the ability to reside in diverse habitats) and a slower tempo of evolution (Bonneaud et al., 2019). Obligate reliance of *Mycoplasma* spp. on host cells and more chronic infections likely explains their propensity to specialize (Citti & Blanchard, 2013; Cohen et al., 2018). More broadly, however, using genetics to infer pathogen sharing, rather than coarser phylogenetic scales (e.g., species complexes or genera), is increasingly showing that many bacterial strains may be more host specific (Withenshaw, Devevey, Pedersen, & Fenton, 2016). The high specialism of bat hemoplasma genotypes thus underscores the importance of using finer phylogenetic scales in the study of infectious disease (Fountain-Jones et al., 2018; Graham, Storch, & Machac, 2018).

Comparative analyses of viruses have suggested that phylogenetically conserved pathogen jumps between species may be a broader generality in the study of disease emergence

(Albery, Eskew, Ross, & Olival, 2019; Luis et al., 2015; Streicker et al., 2010). With few exceptions, our results on hemoplasma genotype sharing between Neotropical bat species are generally consistent with this pattern for a bacterial pathogen. Two cases in which hemoplasmas were shared between more distantly related species included the VBG1 genotype in *Desmodus rotundus* and *Pteronotus fulvus* (Phyllostomidae and Mormoopidae) and the EF1 genotype in *Glossophaga soricina* and *Saccopteryx bilineata* (Phyllostomidae and Emballonuridae). For the latter, both bat species co-roost in the LAR, which suggests an ecological context for pathogen exposure over current timescales. However, other genetic markers (e.g., *rpoB, rpoC, gyrB*) would be necessary to infer contemporary cross-species transmission (Kämpfer & Glaeser, 2012; Volokhov et al., 2012), as analysis of the 16s rRNA gene alone is insufficient for hemoplasma species identification (Volokhov et al., 2012). If hemoplasmas are more likely to specialize rather than expand their range into new and unrelated species, genotype sharing between unrelated bats could represent more transient spillovers (Bonneaud et al., 2019). As specialized pathogens could be more transmissible than generalists (Futuyma & Moreno, 1988; Garamszegi, 2006), species with high prevalence of specialist genotypes could be prioritized for surveillance.

In conclusion, our analysis of a diverse community of bats and their pathogen genotypes identifies several key ecological and evolutionary factors structuring bacterial infection within and between species and provides a starting point for contrasts with such patterns for viruses. Similar to bat viral infections, we found moderate phylogenetic signal in bacterial prevalence. However, these phylogenetic patterns in hemoplasma prevalence were decoupled from those describing bat species centrality in sharing hemoplasma genotypes, such that genotype sharing was generally restricted by bat phylogeny. These findings imply the codivergence of bats and their bacterial pathogens alongside rare and phylogenetically constrained host shifts. More

broadly, our study highlights the importance of using fine phylogenetic scales when assessing host specificity and suggests phylogenetic similarity may play a key role in host shifts for not only viruses but also bacteria. Our work also more broadly contributes to increasing efforts to understand cross-species transmission and epidemiological consequences of bacterial pathogens.

Competing interests

We have no competing interests.

Data accessibility

Individual-level data are available in Dryad (https://datadryad.org/stash/share/7qOGkejyIldAcD9FEZHNNVCc_4k0lmmiABy0EiRwZoo). Bat species trait data are available in Table S2. Hemoplasma sequences are available in GenBank (MH245119–MH245194 and MK353807–MK353892).

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Statement of authorship

DJB, KAS, and AMB collected samples; NBS and MBF coordinated fieldwork; RKP, SA, and DGS supported laboratory analyses; DVV and VEC conducted molecular and phylogenetic analyses; and ADW and DGS assisted with statistical analyses. DJB analyzed data, produced figures, and wrote the manuscript. All authors provided critical review of the manuscript.

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873 **Tables**

874 Table 1. Competing PGLS models predicting hemoplasma infection prevalence (logit-
875 transformed) across the Belize bat community (n=33 species). Models are ranked by ΔAICc with
876 the number of coefficients (k), Akaike weights (w_i), and a likelihood ratio test pseudo-R^2.

Model structure	***k***	**ΔAICc**	w_i	R^2
~ log body mass	2	0.00	0.40	0.24
~ maximum colony size	2	1.71	0.17	0.20
~ roost type	2	2.82	0.10	0.18
~ foraging strata	3	2.88	0.09	0.24
~ roost flexibility	2	3.56	0.07	0.16
~ percent plants in diet	2	4.30	0.05	0.14
~ dietary guild	3	5.46	0.03	0.17
~ log evolutionary distinctiveness	2	5.50	0.02	0.11
~ log aspect ratio	2	5.61	0.02	0.10
~ log annual fecundity	2	6.13	0.02	0.09
~ square-root geographic range size	2	6.27	0.02	0.08
~ 1 (intercept only)	1	6.77	0.01	0.00
~ sample size	2	8.38	0.01	0.02

877

878 Table 2. Hemoplasma genotypes identified from the Belize bat community. Genotypes are given

879 with their bat host species, representative GenBank numbers, and intra-genotype variability.

Genotype	Host species	Representative GenBank number	Intra-genotype sequence variability
VBG1	*Desmodus rotundus, Pteronotus fulvus*[***]	KY932701	99.8
VBG2	*Desmodus rotundus*	KY932678	99.9
VBG3	*Desmodus rotundus*	KY932722	99.6
CS1[**]	*Carollia sowelli*	MK353833	100
CS2[**]	*Carollia sowelli, C. perspicillata*	MH245134	99.7
MR1[**]	*Molossus rufus*	MH245174	99.7
MR2[**]	*Molossus rufus*	MH245151	NA[*]
PPM[**]	*Pteronotus mesoamericanus, P. fulvus*[***]	MH245159	99.9
EF1[**]	*Eptesicus furinalis, Saccopteryx bilineata*[***], *Glossophaga soricina*[***]	MH245147	99.6
EF2[**]	*Eptesicus furinalis*	MH245131	99.9
NM[**]	*Natalus mexicanus*[***]	MK353818	NA[*]
LE[**]	*Lophostoma evotis*	MK353892	99.9
TC1[**]	*Trachops cirrhosus*	MH245145	99.8
TC2[**]	*Trachops cirrhosus*	MK353860	99.8
APH1[**]	*Dermanura phaeotis, D. watsoni, A. lituratus*[***]	MH245132	100
APH2[**]	*Artibeus jamaicensis, A. lituratus, A. intermedius*	MH245187	99.9
APH3[**]	*Artibeus intermedius*	MH245186	99.8
GLS[**]	*Glossophaga soricina*	MK353874	99.5
MYE[**]	*Myotis elegans, Myotis keaysi*[***]	MK353840	100
MYK[**]	*Myotis keaysi*[***]	MH245153	NA[*]
UB[**]	*Uroderma convexum*	MK353869	99.8
PLU[**]	*Platyrrhinus helleri*[***], *Uroderma convexum*[***]	MK353883	99.6
SP[**]	*Sturnira parvidens; A. lituratus*[***]	MH245168	99.5
RHN[**]	*Rhynchonycteris naso*[***]	MK353871	NA[*]
M. moatsii-like 1[****]	*Pteronotus mesoamericanus*[***]	MK353864	NA[*]
M. moatsii-like 2[****]	*Myotis keaysi*[***]	MK353862	NA[*]
M. moatsii-like 3[****]	*Rhynchonycteris naso*[***]	MH245146	NA[*]
M. lagogenitalium-like[****]	*Glossophaga soricina*[***]	MH245140	NA[*]
M. muris-like[****]	*Saccopteryx bilineata*[***]	MH245138	NA[*]

880 *Intra-genotype sequence variability could not be assessed, as only one sequence was identified

881 **Novel hemoplasma genotypes

882 ***Genotypes were detected in only one individual of these bat species

883 ****Non-hemoplasma *Mycoplasma* genotypes

884

885 **Figures and legends**

886 Figure 1. Study sites in northern Belize. The shaded inset shows the location of Orange Walk
887 District. Borders show the boundaries of the LAR (Lamanai Archaeological Reserve) and KK
888 (Ka'Kabish). White and tan shading indicates agricultural and urban development, while dark
889 green shading represents intact forest. Satellite imagery was derived from Google Maps. Stacked
890 bar plots display the relative abundance of each sampled bat family per study site.

891

892 Figure 2. Predictors of hemoplasma infection status. (A) Odds ratios and 95% HDIs from the
893 phylogenetic GLMM. Estimates that do not overlap with 1 (dashed line) are displayed in black.
894 Prevalence and 95% confidence intervals are stratified by (B) bat sex and (C) site per year.

895

896 Figure 3. Predictors of species-level hemoplasma prevalence across the Belize bat community.
897 (A) Clades with significantly different prevalence are highlighted. (B) Results from the top
898 PGLS models predicting prevalence as a function of mass and colony size. Model fit and 95%
899 confidence intervals are shown overlaid with data scaled by sample size; species from the clade
900 identified through phylogenetic factorization are colored as in panel A. Species identified
901 through phylogenetic factorization (*Saccopteryx bilineata* and *Rhynchonycteris naso*) and with
902 larger body mass, colony size, and hemoplasma prevalence (*Desmodus rotundus*, *Molossus*
903 *rufus*, and *Pteronotus mesoamericanus*) are shown to the right (photographs by Brock Fenton).

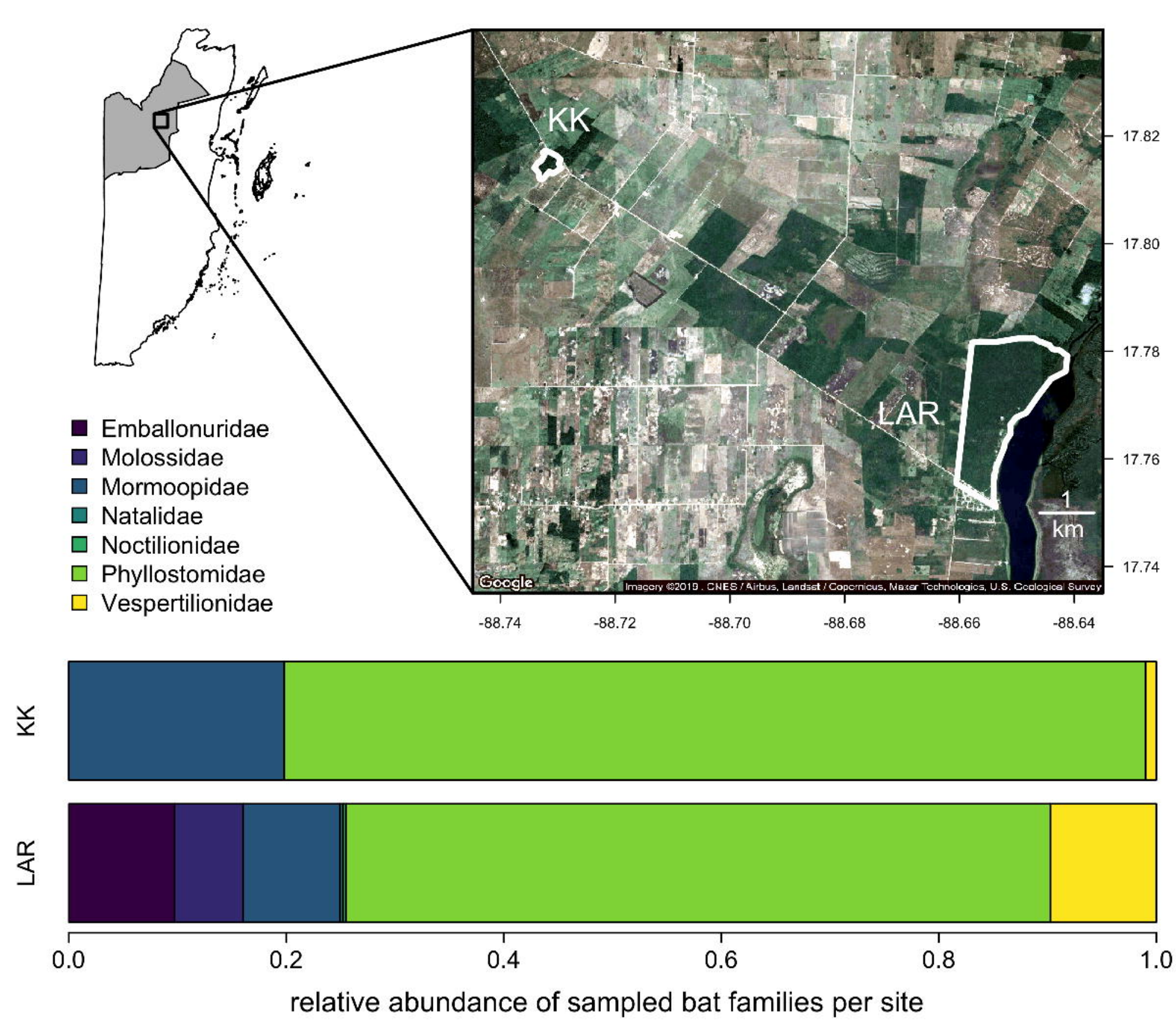
904

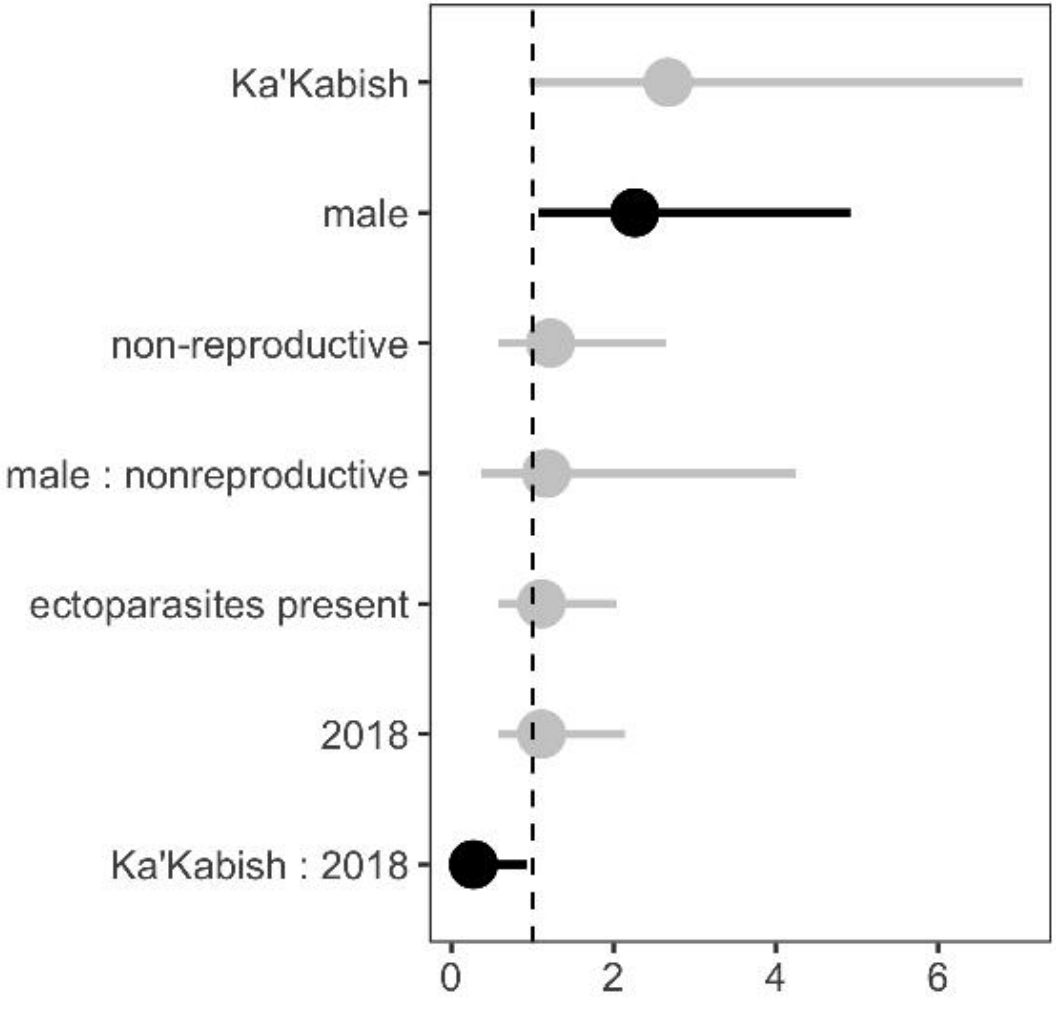
905

Figure 4. Evolutionary relationships between Belize bats and hemoplasma genotypes. The cophylogeny plot shows the bat phylogeny on the left and the hemoplasma genotype phylogeny on the right. Lines display bat–hemoplasma associations and are shaded by the inverse of squared residuals from PACo (i.e., small residuals more indicative of coevolution are dark).

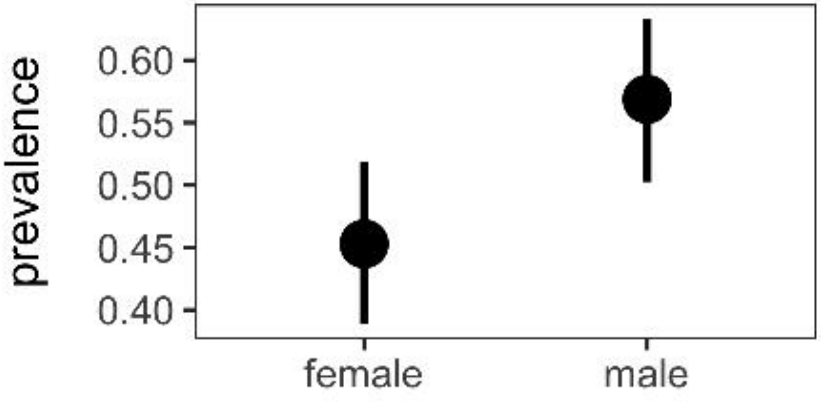
Figure 5. Patterns of hemoplasma genotype sharing across the Belize bat community. (A) Nodes in the genotype network represent bat species (abbreviated by Latin binomials), and edges represent a shared genotype. Nodes are colored by communities identified with the Louvain method and are scaled by the number of individuals per species. (B) The matrix shows pairwise hemoplasma genotype sharing, colored by the number of genotypes shared between bat species.

Figure 6. Phylogenetic patterns in hemoplasma genotype networks for Belize bat species (A) degree and (B) eigenvector centrality. Clades showing significantly different centrality metrics are highlighted, and points are scaled by observed values. Results from the top PGLS models predicting both centrality metrics as a function of bat species traits (C–D). Model fit and 95% confidence intervals are shown overlaid with data scaled by sample size; species from the clades identified through phylogenetic factorization are colored as in panels A–B.

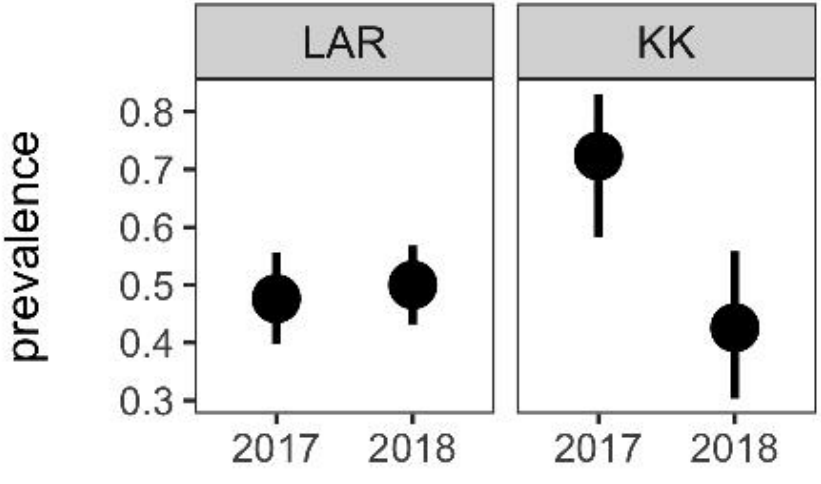

KK
LAR
Emballonuridae
Molossidae
Mormoopidae
Natalidae
Noctilionidae
Phyllostomidae
Vespertilionidae
17.82
17.80
17.78
17.76
17.74
1
km
Google
Imagery ©2019 , CNES / Airbus, Landsat / Copernicus, Maxar Technologies, U.S. Geological Survey
-88.74
-88.72
-88.70
-88.68
-88.66
-88.64
KK
LAR
0.0
0.2
0.4
0.6
0.8
1.0
relative abundance of sampled bat families per site



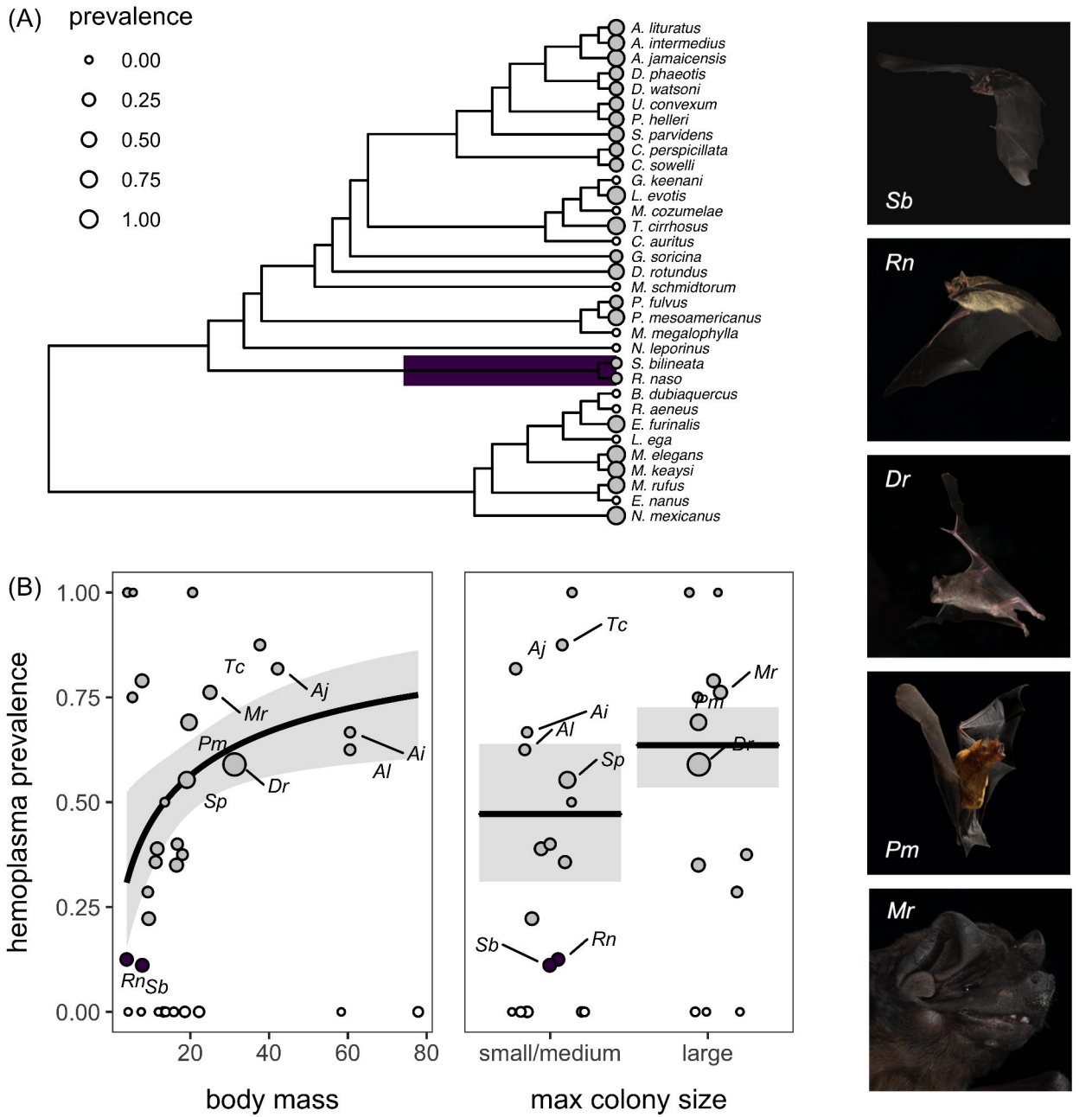
(A) odds of hemoplasma infection

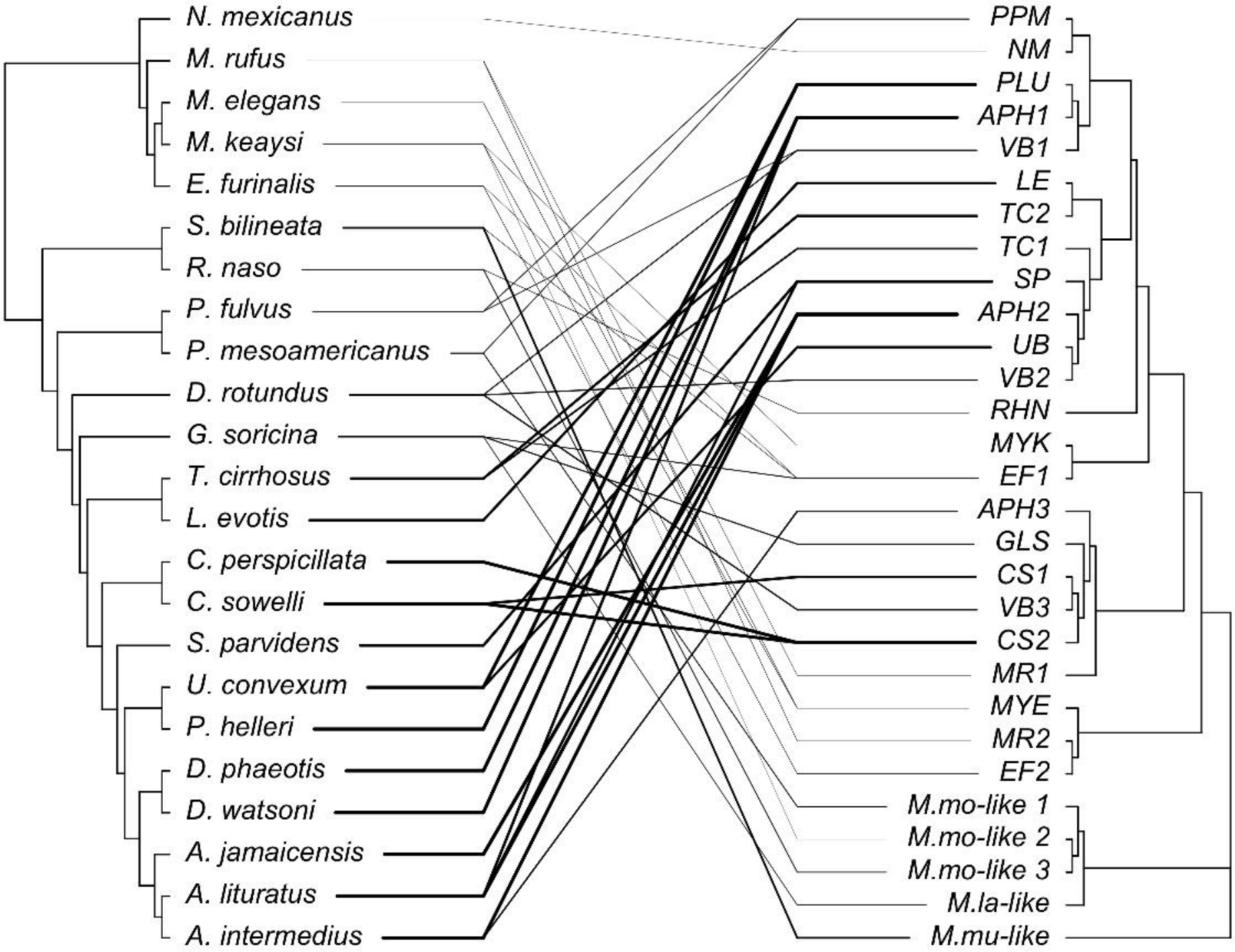


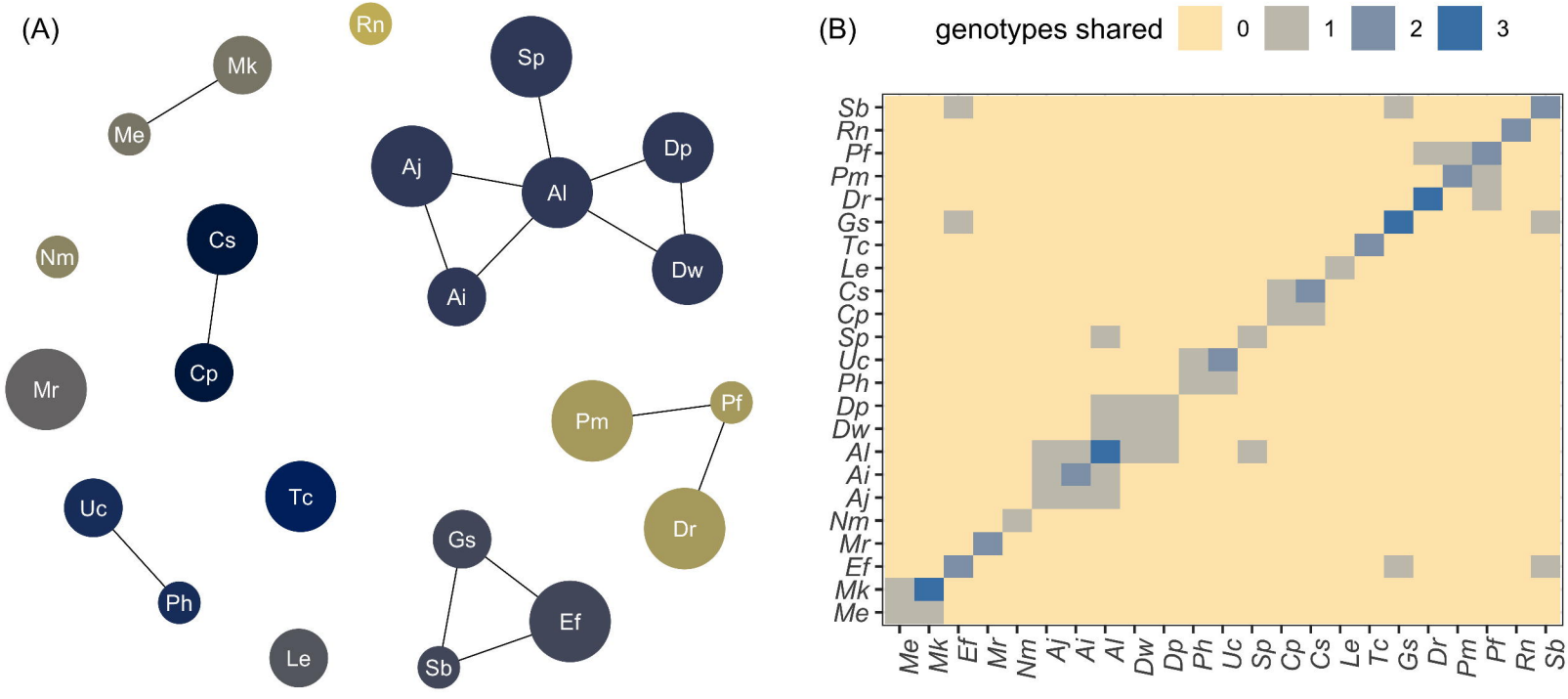
(B) bat sex



(C) sampling year


(A)
prevalence
0.00
0.25
0.50
0.75
1.00
A. lituratus
A. intermedius
A. jamaicensis
D. phaeotis
D. watsoni
U. convexum
P. helleri
S. parvidens
C. perspicillata
C. sowelli
G. keenani
L. evotis
M. cozumelae
T. cirrhosus
C. auritus
G. soricina
D. rotundus
M. schmidtorum
P. fulvus
P. mesoamericanus
M. megalophylla
N. leporinus
S. bilineata
R. naso
B. dubiaquercus
R. aeneus
E. furinalis
L. ega
M. elegans
M. keaysi
M. rufus
E. nanus
N. mexicanus
Sb
Rn
Dr
Pm
Mr
(B)
hemoplasma prevalence
1.00
0.75
0.50
0.25
0.00
Tc
Aj
Mr
Pm
Ai
Al
Dr
Sp
Rn
Sb
20
40
60
80
body mass
small/medium
large
max colony size


N. mexicanus
M. rufus
M. elegans
M. keaysi
E. furinalis
S. bilineata
R. naso
P. fulvus
P. mesoamericanus
D. rotundus
G. soricina
T. cirrhosus
L. evotis
C. perspicillata
C. sowelli
S. parvidens
U. convexum
P. helleri
D. phaeotis
D. watsoni
A. jamaicensis
A. lituratus
A. intermedius
PPM
NM
PLU
APH1
VB1
LE
TC2
TC1
SP
APH2
UB
VB2
RHN
MYK
EF1
APH3
GLS
CS1
VB3
CS2
MR1
MYE
MR2
EF2
M.mo-like 1
M.mo-like 2
M.mo-like 3
M.la-like
M.mu-like


(A)
Rn
Sp
Mk
Me
Aj
Al
Dp
Cs
Nm
Dw
Ai
Cp
Mr
Pm
Pf
Tc
Uc
Dr
Gs
Ef
Ph
Sb
Le
(B)
genotypes shared
0
1
2
3
Sb
Rn
Pf
Pm
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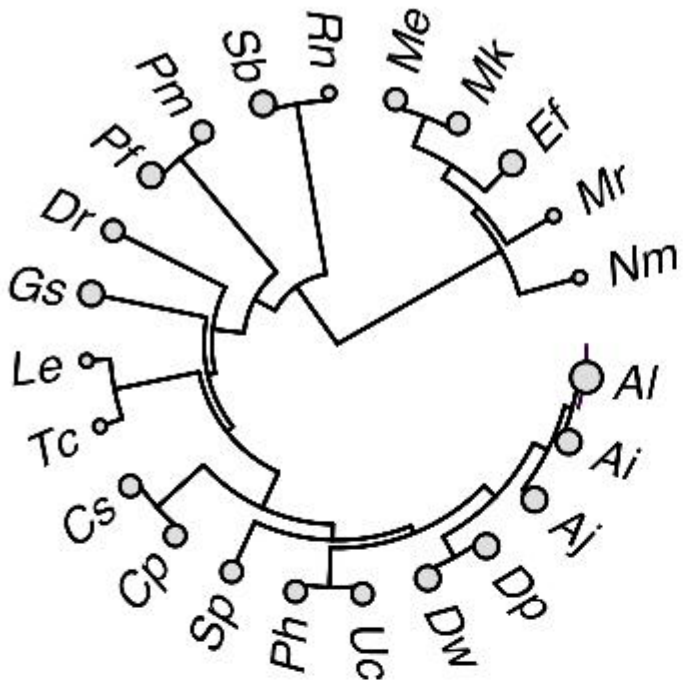
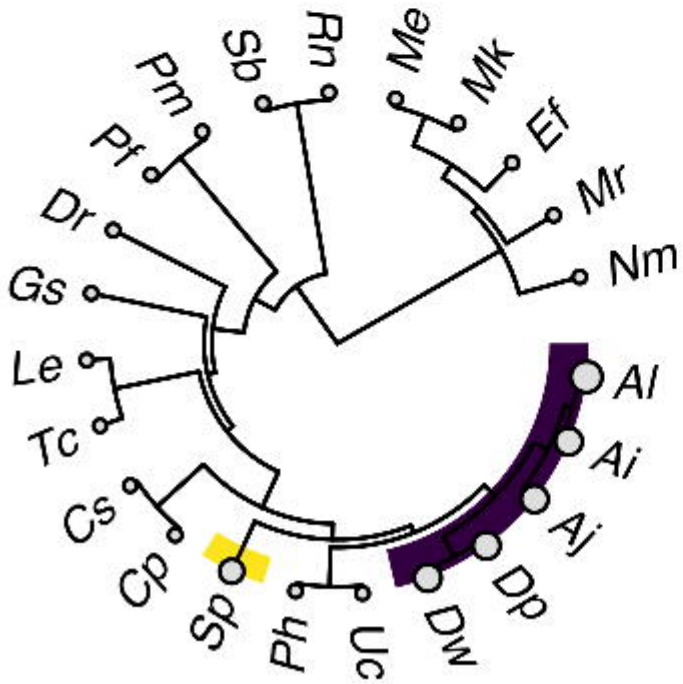
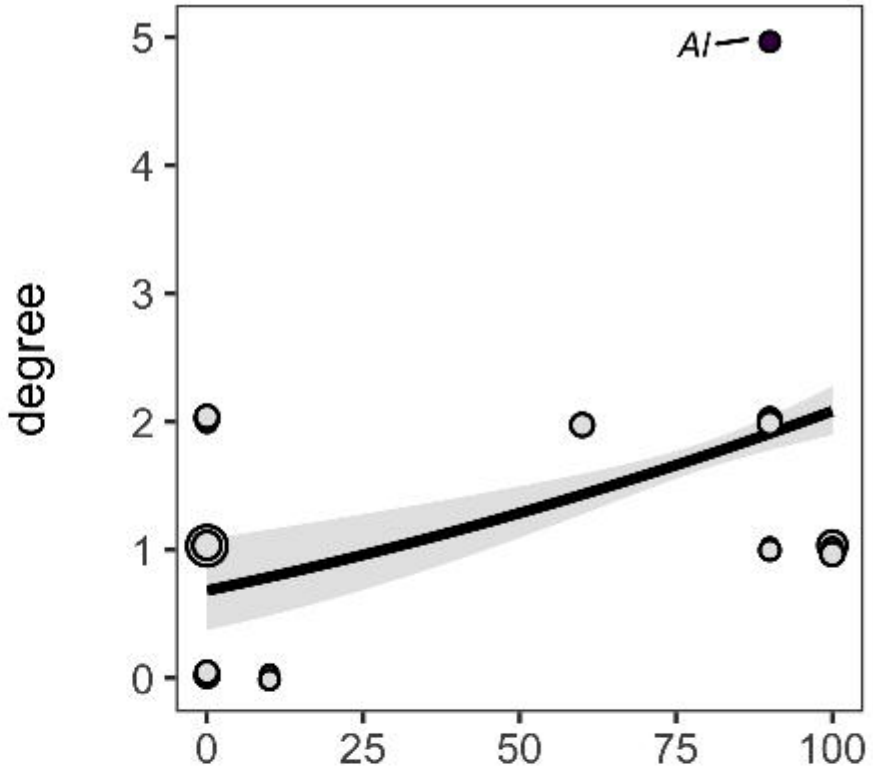


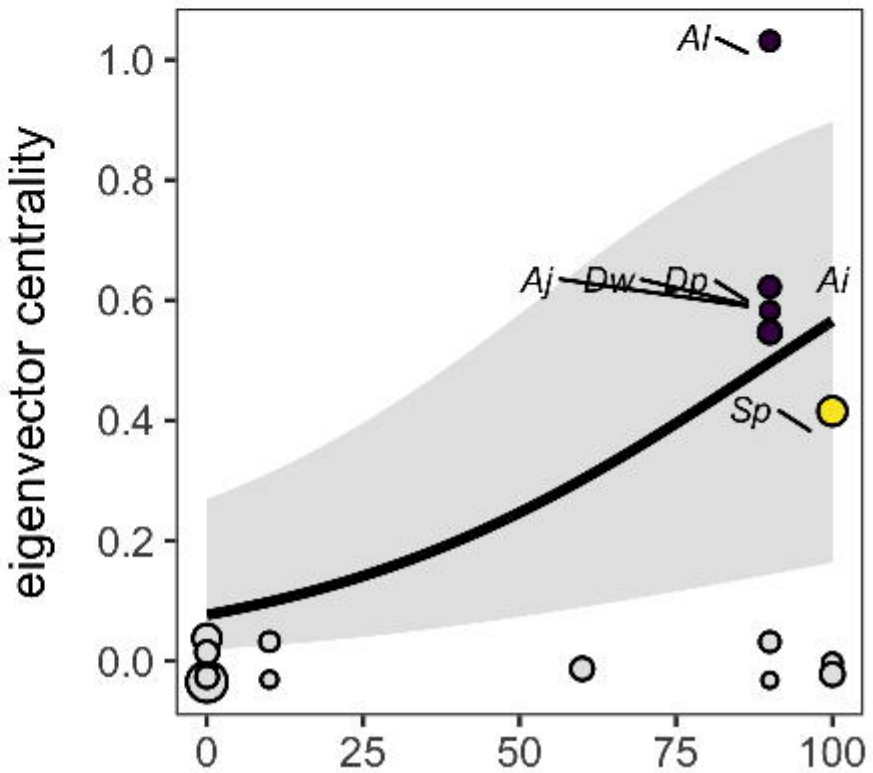
(A) degree



(B) eigenvector centrality



(C) percent plants in diet



(D) percent plants in diet