Integrating population genetic structure, microbiome, and pathogens presence data in *Dermacentor variabilis*

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Tick-borne diseases (TBDs) continue to emerge and re-emerge in several regions of the world, highlighting the need for novel and effective control strategies. The development of effective strategies requires a better understanding of TBDs ecology, and given the complexity of these systems, interdisciplinary approaches are required. In recent years, the microbiome of vectors has received much attention, mainly because associations between native microbes and pathogens may provide a new promising path towards the disruption of pathogen transmission. However, we still do not fully understand how host genetics and environmental factors interact to shape the microbiome of organisms, or how pathogenic microorganisms affect the microbiome and vice versa. The integration of different lines of evidence may be the key to improve our understanding of TBDs ecology. In that context, we generated microbiome and pathogen presence data for *Dermacentor variabilis*, and integrated those data sets with population genetic data, and metadata for the same individual tick specimens. Clustering and multivariate statistical methods were used to combine, analyze, and visualize data sets. Interpretation of the results is challenging, likely due to the low levels of genetic diversity and the high abundance of a few taxa in the microbiome. *Francisella* was dominant in almost all ticks, regardless of geography or sex. Nevertheless, our results showed that, overall, ticks from different geographic regions differ in their microbiome composition. Additionally, DNA of *Rickettsia rhipicephali, R. montanensis, R. bellii,* and *Anaplasma* spp., was detected in *D. variabilis* specimens. This is the first study that successfully generated microbiome, population genetics, and pathogen presence data from the same individual ticks, and that attempted to combine the different lines of evidence. The approaches and pre-processing steps used can be applied to a variety of taxa, and help better understand ecological processes in biological systems.

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ABSTRACT

Tick-borne diseases (TBDs) continue to emerge and re-emerge in several regions of the world, highlighting the need for novel and effective control strategies. The development of effective strategies requires a better understanding of TBDs ecology, and given the complexity of these systems, interdisciplinary approaches are required. In recent years, the microbiome of vectors has received much attention, mainly because associations between native microbes and pathogens may provide a new promising path towards the disruption of pathogen transmission. However, we still do not fully understand how host genetics and environmental factors interact to shape the microbiome of organisms, or how pathogenic microorganisms affect the microbiome and vice versa. The integration of different lines of evidence may be the key to improve our understanding of TBDs ecology. In that context, we generated microbiome and pathogen presence data for *Dermacentor variabilis*, and integrated those data sets with

population genetic data, and metadata for the same individual tick specimens. Clustering and multivariate statistical methods were used to combine, analyze, and visualize data sets. Interpretation of the results is challenging, likely due to the low levels of genetic diversity and the high abundance of a few taxa in the microbiome. *Francisella* was dominant in almost all ticks, regardless of geography or sex. Nevertheless, our results showed that, overall, ticks from different geographic regions differ in their microbiome composition. Additionally, DNA of *Rickettsia rhipicephali, R. montanensis, R. bellii,* and *Anaplasma* spp., was detected in *D. variabilis* specimens. This is the first study that successfully generated microbiome, population genetics, and pathogen presence data from the same individual ticks, and that attempted to combine the different lines of evidence. The approaches and pre-processing steps used can be applied to a variety of taxa, and help better understand ecological processes in biological systems.

INTRODUCTION

As vector-borne diseases continue to emerge and re-emerge in several regions of the world, there is an urgent need for a better understanding of their ecology, including the tripartite pathogen-vector-host relationship, to ultimately develop effective control strategies. Ticks represent an increasing threat to One Health given the range expansion of some species, and the frequent identification of new tick-borne pathogens (Paddock et al. 2016; Eisen et al. 2017). Due to the complex nature of diseases transmitted by ticks, their control requires interdisciplinary studies and collaboration (Murgia et al. 2019).

In recent years, the microbiome (referring to bacteria only throughout the manuscript) of vectors (mostly mosquitoes and ticks) has received much attention. The main reason is that

53 associations between native microbes and pathogens may provide a new promising path towards
54 the disruption of pathogen transmission (Narasimhan & Fikrig 2015; Bonnet et al. 2017). In the
55 case of ticks, microbes can be acquired from the environment (e.g through the spiracles),
56 horizontally (from the host, during blood feeding), or vertically (maternally inherited).
57 Relationships between the tick and a particular microbe or set of microbes can range from
58 mutualistic to parasitic (Casadevall et al. 2011; Bonnet et al. 2017). Microbes can affect tick
59 fitness, vector competence, and pathogen transmission (Bonnet et al. 2017; Budachetri et al.
60 2018). For example, a few maternally inherited microbes have been proposed as primary
61 symbionts necessary for tick success by providing vitamins that are lacking in blood (Smith et al.
62 2015b; Duron et al. 2017; Guizzo et al. 2017). Without these microbes, the ticks' fitness is
63 negatively impacted (Zhong et al. 2007). Other symbionts interact with pathogenic
64 microorganisms, either positively or negatively. High proportions of the endosymbiotic
65 *Rickettsia bellii* inhibit the transmission of the pathogenic *Anaplasma marginale* (Gall et al.
66 2016), and *R. parkeri* is likely excluded from *A. maculatum* by Candidatus *Rickettsia andeanae*
67 (Paddock et al. 2015), presumably through a phenomenon known as interference. On the other
68 hand, cases of facilitation have also been reported; such as a positive relationship between the
69 proportion of the microbiome occupied by *Francisella* endosymbiont (FLE) and the infection
70 level of *F. novicida* (Gall et al., 2016).
71 Even though a considerable amount of information and knowledge has been generated
72 during the last decade or so, microbiome research on ticks is still in its infancy. For example, we
73 still do not fully understand how host genetics and environmental factors interact to shape the
74 microbiome of organisms (Spor et al. 2011; Goodrich et al. 2014; Steury et al. 2019), or how
75 pathogenic microorganisms affect the microbiome and vice versa. Integration of different lines

of evidence may be the key to improve our understanding of TBDs ecology. As highlighted by Griffiths et al. (2018), research exploring the links between, for example, vector genetics, microbiome composition and structure, and pathogen susceptibility may enable a better understanding of the factors governing disease in vulnerable populations. Unfortunately, to the best of our knowledge, there are no such studies on ticks. There are a few studies based on vertebrate taxa that have attempted to incorporate genomic and microbial data with data on environmental variation (Fietz et al. 2018; Griffiths et al. 2018; Steury et al. 2019). Overall, these studies have shown that genetically divergent host populations, exhibited more divergent microbiomes (Smith et al. 2015a; Griffiths et al. 2018; Steury et al. 2019). In amphibians, Griffiths et al. (2018) found that the genetic distance among hosts was correlated with microbial community dissimilarity when controlling for geographic distance. Nevertheless, the same study showed that at the site-level the microbiome did not mirror the host population genetic structure. Steury et al. (2019) investigated populations of Threespine Stickleback and concluded that the microbiome composition was better predicted by fish population genetic divergence than by geographic distance and environment. It is worth noting that the global trends appear to be driven by a subset of the microbiome. In other words, the influence of host genetic factors on the microbiome composition depends on the bacterial taxa in question; host genetics may affect the presence of some microbes, but presence of others may be better explained by, for example, the environment (Fietz et al. 2018). In the same way, differences between populations could be the result of differences in the relative abundance of a small subset of microorganisms (Steury et al. 2019).

The vertebrate results suggest that integrating microbiome and population genetic data may lead to better understanding of the ecology of tick-borne diseases. Do ticks from different

genetic clusters harbor a different microbiome? Do infected ticks belong to a specific genetic cluster? Is the microbiome of infected ticks different from that of non-infected ticks? To start answering these questions, it is paramount to first generate high quality data, and then to develop an appropriated framework to integrate the different lines of evidence. This exploratory study focuses on *Dermacentor variabilis* (Say), a North American tick species that commonly bites humans, and that historically has been implicated in the transmission of several pathogens (Hecht et al. 2019). *Dermacentor variabilis* is also one of the most widely distributed ticks in the United States (Eisen et al. 2017), one for which population genetic data was already available. During this investigation, we first generated high-quality microbiome data and determined the presence/absence of several pathogens. Next, we combined those data sets with an existing population genetics data set for *D. variabilis* (see Lado et al. 2019). All three pieces of data (microbiome, pathogens, and population genetics) were derived from each of the individual ticks included in this study. The goal of this preliminary study was to integrate the different lines of evidence for *D. variabilis* ticks using clustering methods and multivariate statistics to identify trends and patterns of variation. Our expectation was that *D. variabilis* ticks that are genetically more alike (i.e. that belong to the same genetic cluster), will also be more alike at the microbiome level. Additionally, we hypothesized that infected and non-infected ticks would have a subset of microbes in their microbiome that are characteristic for each category.

METHODS

Tick samples

121 The tick specimens employed in this study are the same individual ticks from Lado et al.
122 (2019), with the exception of two specimens for which we did not have enough genetic material.
123 The sample includes 64 adult *D. variabilis* collected from California (n=3), Georgia (n=2),
124 Indiana (n=3), Maryland (n=4), Maine (n=3), Minnesota (n=5), North Dakota (n=1), Ohio
125 (n=31), Oklahoma (n=5), Tennessee (n=5), Virginia (n=1), and Washington (n=1) (Fig. 1). All
126 specimens were wild caught by dragging a 1m x 0.8m cloth thorugh the vegetation, and
127 preserved in 95% ethanol. We collected ticks from vegetation (unfed) to minimize the amount of
128 genetic material from the host. Collection data for the specimens used in this study can be
129 accessed through the Ohio State Acarology Collection (OSAL) online database
130 (https://acarology.osu.edu). Metro Parks permitted collection of tick species throughout the
131 Metro Parks. The Division of State Parks (DNR), Indiana Department of Natural Resources
132 authorized tick collection at Brown County State Park, Clifty Falls State Park, O'Bannon Woods
133 State Park, Monroe Lake, Splinter Ridge Fish and Wildlife Area, Clark State Forest, and
134 Ferdinand State Forest.
135
136 **DNA extraction**
137 Before DNA extraction the ticks were surface sterilized following Lado et al. (2018);
138 specifically, the washes consist on following this procedure twice: commercial bleach 3% for
139 one minute, washed in distilled water, and 95% ethanol for another minute. DNA extractions of
140 individual tick specimens were performed using the QIAgen Blood and Tissue kit following the
141 manufacturers' instructions, with one exception as in Lado et al. (2018): during the incubation in
142 ATL buffer the posterior-lateral part of the tick idiosoma was cut with a scalpel to allow a better
143 penetration to the buffer to the tick's tissues. The cuticle of all ticks was recovered and kept as

144 voucher. OSAL accession numbers are listed in Table 1. Genomic DNA was quantified using a
145 Qubit 3.0 fluorometer; and then aliquoted and kept in the freezer until used.
146
147 **Population genetic data**
148 For the integrative portion of the analysis, we employed all genetic data generated
149 previously, and followed the genetic clusters nomenclature from Lado et al. (2019). Details on
150 how the population genetics data set was generated and analyzed can be found elsewhere (Lado
151 et al. 2019). In brief, ticks were assigned to three different genetic clusters according to their
152 population genetic structure: a generally "western cluster" (n=4), an "eastern cluster" (n=51),
153 and a "northern cluster" (n=9). This clusters usually correspond to the locations where ticks were
154 collected.
155
156 **Detection of Rickettsiales through PCR**
157 PCR screening
158 Tick DNAs were tested by real-time PCR to detect three genera that include pathogenic
159 microorganisms: *Rickettsia*, *Anaplasma*, and *Ehrlichia.* All ticks were initially screened using
160 two real-time assays: 1) a TaqMan Pan-*Rickettsia* assay, which amplifies a portion of the 23S
161 gene for all *Rickettsia* species using primers PanR8-F and PanR8-R (Kato et al. 2013), and 2) an
162 EvaGreen Anaplasmataceae assay that targets a portion of the 16S gene using primers
163 ECHSYBR-F and ECHSYBR-R, amplifying all *Anaplasma* and *Ehrlichia* species (Li et al.
164 2002).
165 Positive tick samples for Pan-*Rickettsia* (23S real-time assay) where further screened to
166 identify the *Rickettsia* species present. Samples were subjected to conventional semi-nested PCR

targeting the *omp*A gene of all spotted fever group *Rickettsia* (Regnery et al. 1991; Eremeeva et al. 2006). PCRs were performed using 1 μM of each primer (Rr190.70, Rr190.602, Rr190.701), 10 μL of Taq PCR Master Mix (QIAGEN), 2 μL of sample DNA in the primary reaction or 2 μl of the primary reaction product in the secondary reaction, and water to bring the final reaction volume to 20 μl. Positive samples were processed as described below to identify species present. We also performed a *R. bellii* specific TaqMan assay targeting *glt*A, the citrate synthase gene (Hecht et al. 2016) for all positive samples for Pan-*Rickettsia*.

All real-time PCRs were performed in duplicate on a BioRad CFX 96 thermal cycler using 4 μL of template DNA in a final reaction volume of 25 μL for the Pan-*Rickettsia* and 20 μL for both the Anaplasmataceae and *R. bellii*-specific assays. We considered samples positive if one of the duplicates had a cycle threshold (Ct) <40. Two sets of negative controls and one set of positive controls were included on each plate, where water was used as the negative non-template control and DNA from cultured *R. rickettsii*, *E. canis*, or an *R. bellii* plasmid were used as positive controls, depending on the assay (Hecht et al. 2019).

Amplicon purification and sequencing

Amplicons from *omp*A semi-nested PCR were visualized on 1.5% agarose gels using ethidium bromide. Amplicons were extracted and purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Products were bidirectionally sequenced using a BigDye Terminator v3.1 kit on an ABI 3500 genetic analyzer (Applied BioSystems, Carlsbad, CA) and assembled using Geneious version 7.0.4. (http:// geneious.com, Kearse et al. 2012). The nucleotide BLAST tool of the NCBI GenBank database was employed to compare the amplicon sequences to those sequences available in the database. Positive amplicons from the Anaplasmataceae assay were also sequenced following the above-mentioned procedure.

Microbiome

16SrDNA library preparation and amplicon sequencing

Genomic DNA samples, along with two negative controls, were taken to a final concentration of 10 ng/µl, and shipped to Argonne National Laboratory for library preparation and sequencing following standard procedures. The two negative controls correspond to a extraction blank and library blank *sensu* Kim et al. 2017. The primer pair 515F/926R (Walters et al. 2016) was employed to amplify the V4-V5 variable regions of the 16S rDNA gene, and then the amplicons were sequenced on a MiSeq illumina platform, paired-end 251bp reads. The obtained reads were demultiplexed using MiSeq Reporter.

Quality filtering, OTU picking, taxonomic assignments, and diversity calculations

Data were initially filtered as previously described in Lado et al. (2018). Specifically, after demultiplexing, Cutadapt (Martin 2011) was used to do an initial quality filter of the reads (threshold Q10), and to trim the adaptors if they were present in the filtered reads. Once the reads passed the initial filters, the QIIME 1.9.1 (Caporaso et al. 2010) pipeline was employed to: assemble the reads; cluster the reads (97% threshold); and to assign taxonomy. Open reference OTUs (operational taxonomic units) picking using uclust, and taxonomic assignment using the Greengenes (DeSantis et al. 2006) and Silva132 (Quast et al. 2013) data bases. An alignment of representative sequences was used as input to generate a tree in FastTree (Price et al. 2009); as well as to construct OTU tables for each of the taxonomic level (i.e genus, family). OTUs abundant in the negative controls and suspected as contaminants were removed in R using the *decontam* package (Davis et al. 2018).

Statistical analysis: integrating individual data sets

OTU tables were imported into R, and finer filtering was performed. First, we eliminated all OTUs that appeared in no more than two samples. Then, with the reduced data, we further eliminated OTUs with less than 0.5% relative abundance in all ticks. The dimensions of the data were therefore substantially reduced. Metadata was then added to the tables, including coordinates, sex, genetic cluster they belong to (following Lado et al. 2019), and the presence of: *Rickettsia*, *Anaplasma* or *Ehrlichia* species for each tick specimen. Additionally, ticks were assigned to three geographic regions following Lado et al. 2019: eastern, western, and northern.

Clustering approach

To determine how samples are related to one another when integrating all variables, a hierarchical clustering approach was taken. In this analysis the variables included were the filtered OTU table, tick ID, sex, genetic cluster, location (as coordinates), and presence of *Rickettsia* or Anaplasmataceae agents. Hierarchical clustering was performed in R and the number of clusters was set to three (k=3), which is the number of genetic clusters. We employed Gower's distance (Gower 1971) as the measure of dissimilarity between samples because it is compatible with mixed data types (quantitative, nominal, and binary variables). We visualized the clustering results through the dendrogram generated by the hierarchy and demonstrated sample organization in space by using the first two principal components of all the filtered OTUs and location coordinates.

As a further exploration, we evaluated how well our clustering matched with the genetic clusters by the concordance index (C-index), which is an internal validation measure of goodness of matching. We also employed methods to determine the optimal number of clusters from the data. This allowed the comparison between the predicted number of clusters (k=3), and the

number of clusters determined inherently from the data set. For this purpose, two methods were used: the average silhouette and the elbow method. The former method computes the average silhouette of observations for different values of *k*. The optimal number of clusters is the one that maximizes the average silhouette over a range of possible values for k (Kaufman & Rousseeuw 1990). On the other hand, the elbow method looks at the total within-cluster sum of squares (wss) as a function of the number of clusters. The optimal *k* will be the smallest one such that adding another cluster doesn't improve much better the total wss.

Ordination methods

Once the data was integrated, ordination methods were used to visualize the samples in space. We used both non-metric multidimensional scaling (NMDS) and Principal coordinates analyses (PCoA) to determine if there was any patterning within the data. Both techniques reduce the high dimensional data into a two-dimensional representation. All analysis and graphs were done in R, employing the *phyloseq* package (McMurdie & Holmes 2013).

Test for significance for different variables

We used Welch's t-test to detect if the relative abundance of a specific species of microorganism differed across host sex or between genetic clusters. Such tests were shown to be flexible and robust even when samples sizes were unbalanced and group variances were unequal (Delacre et al. 2017).

To compare microbial beta diversity across treatments (sex, genetic cluster, and region) we calculated the distance between microbial communities by using two metrics. The first one, Bray-Curtis distance, considers the relative abundance of bacteria, while the second, Jaccard's distance (or dissimilarity index), measures differences in presence/absence of Bacteria. All distances were calculated using the *vegan* package (Oksanen et al. 2019) in R. We then

partitioned the matrices by treatment and performed permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) to determine if beta diversity differed between treatments. PERMANOVA calculations were performed using the *adonis* function in the vegan package with 999 permutations. For those treatments that included more than two groups, a post hoc test was performed to identify the pairs of groups between which the bulk of differences occurred. For that purpose, we employed the *paiwise.adonis* function from the same package.

RESULTS

Detection of Rickettsiales through PCR

DNA belonging to the genus *Rickettsia* was detected in 15.6% (10/64) of the screened ticks including *R. montanensis* (n=6, 9.3%); *R. rhipicephali* (n=2, 3.1%); and *R. bellii* (n=2, 3.1%). *Rickettsia bellii* was detected only in specimens collected from the western U.S. *Anaplasma* DNA was found in a single tick (1.6%), collected in Oklahoma. The DNA sequence obtained was identified as "Uncultured *Anaplasma* sp. clone 15-3642 16S ribosomal RNA gene, GenBank number MG429812". The sequence generated in this study was 100% identical to MG429812, and the query coverage was also 100%, corresponding to bases 23 to 168. None of the ticks were infected with *Ehrlichia* species. A summary of PCR results and metadata for each tick specimen is presented in Table 2.

Microbiome

Microbiome data were successfully generated for all ticks. However, some issues were encountered when attempting to merge forward and reverse reads (only a minimal fraction of the reads merged correctly). Because of that and given the large amount of data obtained, only

forward reads (R1) were employed for all downstream analysis. It is possible that the difficulties encountered when merging the reads is due to the primers pair selected, as other research groups have faced similar issues with these primers for different taxa (Argonne National Lab personal communication). The number of Illumina forward (R1) reads obtained was 2,131,680. After quality filtering the number of reads retained was 2,129,331 with an average length of 251 bp. The average number of sequences per sample was 32,795 (minimum = 9,331; maximum = 44,533). The number of reads for the extraction blank was 2349, considerably lower than all samples; and no reads were generated from the library blank.

A total of 41 orders of bacteria were identified (13 remaining after filtering), and three of them were dominant: Rickettsiales, Legionellales, and Enterobacteriales (Fig. 2A). At the genus level, 61 taxa were detected (Supplementary file 1). Five taxa were removed since their relative abundance was greater in the negative control than in tick samples. Of the 56 genera remaining, 36 were eliminated during the filtering steps according to minimum relative abundance and the number of samples (see Methods section for details). Thus, 20 genera were kept for downstream analyses (Supplementary file 1). Three taxa were highly abundant at the genus level: *Rickettsia* spp., *Francisella* spp., and Enterobacteriaceae "other". (Fig. 2B). *Francisella spp.* were present in all ticks, with a relative abundance ranging from 0.4 to 100% (mean = 81%, median = 98.5%). *Rickettsia* was detected in 12 (18.8%) of the ticks. In all cases where *Rickettsia* spp. had a relative abundance of at least 0.5% by microbiome analysis, tick specimens were also PCR positive, thus microbiome and PCR results are overall, congruent (Table 2). *Rickettsiella*, an intracellular endosymbiont of arthropods (Leclerque 2008), was found in a tick from Ohio (1/64; tick ID 119572D) with a relative abundance of 11%.

Statistical analysis: integrating individual data sets

Clustering approach

The dendrogram resulting from hierarchical clustering showed that the two most distinct samples (clustering together) were from the same location and collection event: Fernald Preserve, Hamilton Co., Ohio (110559-1 and 110559-2) (Fig. 3). The second clade grouped two samples from CA, the two samples collected in Lake Co (115102A,B). The next pair of samples corresponded to the two remaining samples from the west coast, a sample from Napa Co, CA (115101C); and one from Whitman Co, WA (115105B). These two samples were closely associated with a clade that included all Northern samples. Lastly, all Eastern samples clustered together, without any internal grouping according to geography (Fig. 3).

No differences were evident upon visualization of community membership or structure. PCA did not show any specific clustering pattern between samples (Supplementary file 2). In fact, most of the ticks were clustered in a single aggregate. The ticks that clustered somewhat separated were firstly the two that also appeared separated in the clustering analysis (110559-1 and 110559-2); and a tick from Ohio, 119241-3. It is important to note that the first two principal components explained only approximately 40% of the variance present in our sample.

As for k , the optimal number of clusters, silhouette method and elbow method suggested $k = 2$ and $k = 4$ respectively (Supplementary file 3). Both values were close to the number of genetic clusters, which was also the number of clusters we used in the analysis. The C-index between the predicted clusters and genetic clusters was 0.61, indicating some weak but nonnegligible consistency between these two pairs.

Ordination methods

When visualizing the samples in the space through NMDS (stress value 0.064), the

328 samples were spread out without any specific pattern (Fig. 4A). In PCoA with Gower's distance
329 as metric, most samples appeared in a single tight aggregate, while a few others dispersed
330 randomly along the axes (Fig. 4B).
331 Test for significance for different variables
332 The number of bacterial genera found in ticks belonging to the Eastern genetic cluster
333 was higher than that of the Western, and Northern clusters. The genus *Francisella* was dominant
334 in almost all the ticks, regardless of geography. Its relative abundance did not differ significantly
335 between males and females (p-val 0.8); nor between ticks belonging to different genetic clusters
336 (Eastern vs Western p-val 0.57, Eastern vs Northern p-val 0.96, Northern vs Western p-val 0.60)
337 (Fig. 5). The relative abundance of this genus was also not significantly different between
338 infected and uninfected ticks (p-val 0.14), although it was generally lower in infected ticks.
339 Infected ticks were defined as those that were PCR positive for either *Anaplasma* or *Rickettsia*
340 species.
341 *Rickettsia* relative abundance did not significantly differ between males and females (p-
342 val 0.44), nor between ticks belonging to different genetic clusters (Northern vs. Eastern p-val =
343 0.26; Northern vs. Western p-val = 0.72; and Eastern vs. Western p-val = 0.45) (Fig. 5).
344 *Rickettsia rhipicephali* and *Anaplasma* spp. were present only in ticks belonging to the Eastern
345 genetic cluster, and *R. montanensis* was present in ticks belonging to the Northern and Eastern
346 clusters. *Rickettsia bellii* was found infecting ticks from the Western and Northern genetic
347 clusters, although of these ticks were collected in the Western region (Fig 6). One of the ticks
348 positive for this species was 115101C, a tick collected in California but with the genetic make-up
349 corresponding to Northern ticks.
350 Overall, microbial beta diversity differed between regions (p-val <0.05), but not between

351 sexes ($p = 0.63$) when using Bray-Curtis distance. Post hoc tests indicated that ticks collected in
352 the Western region harbored a different microbiome from those collected in the Eastern or
353 Northern regions ($p < 0.05$). In the case of presence/absence of Bacteria (Jaccard distance), the
354 results were the same. A significant difference between ticks collected from different regions
355 was detected ($p< 0.05$), and the post hoc tests showed that the differences were significant
356 between the Western and both the Eastern and Northern regions.

357 The Adonis test for genetic clusters showed that there were differences ($p < 0.05$) only
358 when considering relative abundance of bacterial taxa (Bray-Curtis distance). When the pairwise
359 Adonis test was performed to determine what genetic clusters differed in microbiome structure
360 ($p <0.05$) between Eastern and Western genetic clusters, but its correction was not (plFDR
361 0.096). Eastern and Western clades are genetically, the two more distant (Lado et al. 2019).
362

363 **DISCUSSION**

364 This work corresponds, to the best of our knowledge, to the first attempt in tick research
365 to integrate population genetic, microbiome, and pathogens presence data to better understand
366 the ecology of TBDs. To optimize comparisons, it was our goal to generate those three pieces of
367 data for each of the individual ticks included in the data set. We show that this is possible, even
368 when techniques based on next generation sequencing require high quantities of high quality
369 DNA.

370 We developed an effective pre-processing and processing procedure for researchers
371 interested in microbiomes of small organisms, or parts of organisms with limited amounts of
372 genetic material. This was motivated by a need for consensus regarding the pre-processing of
373 ticks for generating microbiome data. For example, it is likely that many of the incongruencies

between microbiome studies arise from differences in approaches to decontamination of the ticks' surface. Several studies in the literature performed surface sterilization of the ticks before DNA extraction (e.g. Lado et al. 2018; Trout Fryxell and DeBruyn 2016), whereas others did not (e.g. Clow et al. 2018); and the differences in bacterial communities may be a reflection of extensive environmental "contamination" in the latter. Even between studies that "washed" the ticks, comparisons need to be done cautiously, since not all sterilization methods are equally effective. The incorporation of bleach appears necessary for effective decontamination (Binetruy et al. 2019). Increased consistency in results of microbiome studies of ticks requires some community consensus related to the generation and analysis of microbiome data. This is especially important for non-model organisms such as ticks, given the "noise" (Alpha and Beta diversity inflation) generated by the presence of environmental microorganisms.

Consistent with most other studies of tick microbiomes (Van Treuren et al. 2015; Gall et al. 2016; Chicana et al. 2019), our analysis showed that the microbiome of many species is heavily dominated by a few genera, while the microbiomes of others, such as *Ixodes angustus* (Chicana et al. 2019) and *Haemaphysalis lemuris* (Lado et al. 2018), are more diverse. The microbiome of *D. variabilis* is dominated by *Francisella* (Chicana et al. 2019; Travanty et al. 2019; current study). Clow et al. (2018) presented, to some extent, opposing results (far higher microbial diversity), but they analyzed only 9 specimens, and the ticks were not surface sterilized. These results casts some doubt on the concept of a "core microbiome". For example, Chicana et al. (2019) report a *D. variabilis* core microbiome as composed by *Francisella*, *Sphingomonas*, and *Methylobacterium*. On the other hand, Travanty et al. (2019) reported a *D. variabilis* core microbiome that includes *Francisella* spp., *Sphingomonas* spp., *Delftia* spp., and *Hymenobacter* spp. Our study recovered all of these taxa but only *Fransicella* spp. is nearly

universally present. *Francisella* has been well established as endosymbiont and dominant in *D. variabilis*, as well as other *Dermacentor* species. All other taxa reported as "core microbiome" are not consistent across studies (e.g. Chicana et al. 2019; Clow et al. 2018; Rynkiewicz et al. 2015; Travanty et al. 2019). Thus, the true "core" of taxa overlapping across studies, can be reduced to *Francisella* only. The concept of "core microbiome" is generally used in microbiome research to refer to a suite of microbes, and not to refer to only one taxon. The utility of the concept of "core microbiome" for *D. variabilis* is therefore unclear.

Recent studies focusing on the pathogen transmission by *D. variabis* and on its role as vector of human diseases have consistently reported low prevalence of infections with known pathogenic microorganisms. For example, *R. rickettsii*, is usually present in ~1% of the ticks analyzed e.g. (Hecht et al. 2019). The results obtained in the present study are consistent with these literature results, and show an overall *Rickettsia* spp. prevalence of 17.2%, including *R. montanensis, R. bellii,* and *R. amblyommatis* infections. *Rickettsia rickettsii* DNA was not found in any of the ticks analyzed. We detected DNA of *Anaplasma* spp. in one tick sample collected in Oklahoma. Its DNA sequence matched 100% with GenBank sequences submitted by researchers at the CDC Fort Collins, who isolated the agent from a human blood sample. No additional information is available in the literature about this case. This study comprises the first report of that specific bacterial agent in *D. variabilis*. However, an *Anaplasma* spp. *bovis*-like agent has been previously reported in *D. andersoni* ticks from Canada (Dergousoff & Chilton 2011; Chilton et al. 2018). Unfortunately, the region of 16S amplified differs between our study and that of *D. andersoni* (Dergousoff & Chilton, 2011), making it difficult to determine if the sequences correspond to the same specific agent. Regardless, it appears that uncharacterized *Anaplasma* agents are circulating in nature (ticks and humans), and their characterization,

420 together with the determination of whether or not they are capable of causing disease in humans
421 should be further explored. Furthermore, due to the finding of this agent's DNA in a *D.*
422 *variabilis* sample in this study, the role of *D. variabilis* as potential vector should be considered
423 and further explored.

424 With respect to the primary goal of this preliminary study, the integration of the different
425 types of data was done successfully, although the interpretation of the results is somewhat
426 challenging. The latter problem is most likely caused by to the low levels of genetic diversity and
427 moderate levels of population structure (see details in Lado et al. 2019), and a microbiome
428 highly dominated by a few taxa. As a result, ordination methods failed to show patterns of
429 variation across different groups. Nonetheless, the dendrogram resulting from the clustering
430 analysis, in which all lines of evidence and geographic location were considered, was largely
431 congruent with the observations at the population genetic level. Thus, the addition of
432 microbiome, pathogen presence, ticks sex, and geographic location led to conclusions that are, in
433 general, consistent with ticks genetics. All Eastern samples clustered together (with the exception
434 of two samples from Hamilton Co, OH), and separated from both Western and Northern
435 samples. The distinctiveness and separation of those two samples from Hamilton Co, OH reflects
436 their distinctiveness at the microbiome level (Fig. 2). The fact that both samples appeared very
437 similar to all other Eastern samples at the population genetic level (Lado et al. 2019), supports
438 that hypothesis. It is possible that these two specimens were not well surface sterilized, or that
439 the environment at that collection location is different.

440 The higher number of bacterial genera found in ticks belonging to the Eastern genetic
441 cluster when compared to either Northern or Western ticks, is likely a product of the higher
442 number of ticks analyzed, and the greater geographic area covered. Even though in the broader

443 sense the microbiome is not diverse (dominated by three genera), it differed between the three
444 main geographic regions: Eastern, Western, and Northern. Statistical results looking at the beta
445 diversity between genetic clusters are less clear: initial test demonstrated a difference in the
446 microbial communities between genetic clusters, but pairwise tests with their corresponding
447 corrections failed to reach the same result. This may be a result of one of the study's limitations,
448 the number of samples. Thus, these findings should be further explored using more samples, and
449 more samples per genetic cluster. From our preliminary analyses, geography explains the
450 differences in the microbial communities better than host genetics. This could be the result of
451 certain microorganisms occurring only in certain geographic areas.
452 Despite challenges arising during the interpretation of the results, the integration of lines
453 of evidence and metadata, revealed that Eastern ticks can be separated from Northern and
454 Western ticks. It can also be noted that some *Rickettsia* species were associated with a certain
455 geographic area. For example, *R. bellii* was found only in samples collected in CA, a finding
456 consistent with that reported in Hecht et. al. (2019). It seems possible that *R. bellii* is more
457 common along the west coast, although more samples should be analyzed to confirm this. And
458 while the two *R. bellii* positive tick samples were collected in CA, one of them (ID 115101C) did
459 not belong to the Western genetic cluster; it belonged to the Northern cluster. From the host
460 genetics perspective, this tick is more similar to ticks from Northern locations; whereas from the
461 rickettsial agents perspective, it is more similar to Western samples. Looking at the microbiome
462 composition, this tick could belong to any geographic region. This underscores the value of
463 integrating different types of data when thinking about disease ecology.
464 The approaches taken during this study, both to generate and analyze data, can be applied
465 to a wide variety of taxa, and groups of organisms. It is likely that in organisms with a higher

level of variation, the clustering methods will be more informative, and their interpretation more straightforward. Therefore, the approaches used herein have potential, and could greatly improve future studies looking at different aspects of diseases ecology. In the particular case of *D. variabilis,* the approaches used herein failed to detect clear tendencies or patterns. This was likely due to the low levels of variation (both in the microbiome and population genetics), and the very low prevalence of pathogenic microorganisms.

CONCLUSIONS

In conclusion, this is the first study that successfully generated microbiome, population genetics, and pathogens presence data for the same individual ticks. General methodologies and pre-processing steps are replicable, and applicable to different groups of organisms across the tree of life. This work also comprises, at present, one of the few studies aiming at integrating population genetics and microbiome data to better understand ecological processes and disease, and it is the first one to do so for ticks. The integration of different lines of evidence allows a more holistic approach; and clustering and ordination methods are very helpful to summarize and visualize the results. Finally, this study comprises the first report of "Uncultured *Anaplasma* sp. clone 15-3642" in *D. variabilis*. This agent was previously isolated from human blood, and it is important to determine if it is capable of causing disease in humans, and if so, the role of *Dermacentor* ticks as potential vectors.

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REFERENCES

Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology **26**:32–46.

Binetruy F, Dupraz M, Buysse M, Duron O. 2019. Surface sterilization methods impact measures of internal microbial diversity in ticks. Parasites and Vectors **12**:268.

Bonnet SI, Binetruy F, Hernández-Jarguín AM, Duron O. 2017. The Tick Microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. Frontiers in Cellular and Infection Microbiology **7**:1–14.

Budachetri K, Kumar D, Crispell G, Beck C, Dasch G, Karim S. 2018. The tick endosymbiont Candidatus *Midichloria mitochondrii* and selenoproteins are essential for the growth of *Rickettsia parkeri* in the Gulf Coast tick vector. Microbiome **6**:141.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez Peña A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR,

512 Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010.
513 QIIME allows analysis of high-throughput community sequencing data. Nature Methods
514 **7**:335–336.
515 Casadevall A, Fang FC, Pirofski L anne. 2011. Microbial virulence as an emergent property:
516 Consequences and opportunities. PLoS Pathogens **7**:e1002136.
517 Chicana B, Couper LI, Kwan JY, Tahiraj E, Swei A. 2019. Comparative microbiome profiles of
518 sympatric tick species from the far-western United States. Insects **10**:1–12.
519 Chilton NB, Dergousoff SJ, Lysyk TJ. 2018. Prevalence of *Anaplasma bovis* in Canadian
520 populations of the Rocky Mountain wood tick, *Dermacentor andersoni.* Ticks and Tick-
521 borne diseases **9**:1528-1531.
522 Clow KM, Weese JS, Rousseau J, Jardine CM. 2018. Microbiota of field-collected *Ixodes*
523 *scapularis* and *Dermacentor variabilis* from eastern and southern Ontario, Canada. Ticks
524 and Tick-borne Diseases **9**:235–244.
525 Davis NM, Proctor D, Holmes SP, Relman DA, Callahan BJ. 2018. Simple statistical
526 identification and removal of contaminants sequences in marker-gene and metagenomics
527 data. Microbiome **6**:226.
528 Delacre M, Lakens D, Leys C. 2017. Why psychologists should by default use welch's t-Test
529 instead of student's t-Test. International Review of Social Psychology **30**:92–101.
530 Dergousoff SJ, Chilton NB. 2011. Novel genotypes of *Anaplasma bovis*, "Candidatus
531 Midichloria" sp. and Ignatzschineria sp. in the Rocky Mountain wood tick, *Dermacentor*
532 *andersoni*. Veterinary Microbiology **12**;150(1-2):100-106.
533 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P,
534 Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and

workbench compatible with ARB. Applied and environmental microbiology **72**:5069–72.

Duron O, Binetruy F, Noël V, Cremaschi J, McCoy KD, Arnathau C, Plantard O, Goolsby J, Pérez de León AA, Heylen DJA, Van Oosten AR, Gottlieb Y, Baneth G, Guglielmone AA7, Estrada-Peña A, Opara MN, Zenner L, Vavre F, Chevillon C. 2017. Evolutionary changes in symbiont community structure in ticks. Molecular Ecology **26**:2905–2921.

Eisen RJ, Kugeler KJ, Eisen L, Beard CB, Paddock CD. 2017. Tick-borne zoonoses in the United States: Persistent and emerging threats to human health. ILAR Journal **58**:319–335.

Eremeeva ME, Bosserman EA, Demma LJ, Zambrano ML, Blau DM, Dasch GA. 2006. Isolation and identification of *Rickettsia massiliae* from *Rhipicephalus sanguineus* ticks collected in Arizona. Applied and Environmental Microbiology **72**:5569–5577.

Fietz K, Rye Hintze CO, Skovrind M, Kjærgaard Nielsen T, Limborg MT, Krag MA, Palsbøll PJ, Hestbjerg Hansen L, Rask Møller P, Gilbert MTP. 2018. Mind the gut: Genomic insights to population divergence and gut microbial composition of two marine keystone species. Microbiome **6**:82.

Gall CA, Reif KE, Scoles GA, Mason KL, Mousel M, Noh SM, Brayton KA. 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. The ISME Journal **10**:1846–1855.

Goodrich JK, Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. 2014. Human genetics shape the gut microbiome. Cell **159**(4):789-799.

Gower JC. 1971. A General Coefficient of Similarity and Some of Its Properties. Biometrics **27**:857–871.

Griffiths SM, Harrison XA, Weldon C, Wood MD, Pretorius A, Hopkins K, Fox G, Preziosi RF,

558 Antwis RE. 2018. Genetic variability and ontogeny predict microbiome structure in a
559 disease-challenged montane amphibian. ISME Journal **12**:2506–2517.
560 Guizzo MG, Parizi LF, Nunes RD, Schama R, Albano RM, Tirloni L, Oldiges DP, Vieira RP,
561 Oliveira WHC, Leite MS, Gonzales SA, Farber M, Martins O, Vaz IDS Jr, Oliveira PL.
562 2017. A *Coxiella* mutualist symbiont is essential to the development of *Rhipicephalus*
563 *microplus*. Scientific Reports **7**:17554.
564 Hecht JA, Allerdice MEJ, Dykstra EA, Mastel L, Eisen RJ, Johnson TL, Gaff HD, Varela-Stokes
565 AS, Goddard J, Pagac BB, Paddock CD, Karpathy SE. 2019. Multistate Survey of
566 American Dog Ticks (*Dermacentor variabilis*) for *Rickettsia* Species. Vector-Borne and
567 Zoonotic Diseases **19**:652–657.
568 Hecht JA, Allerdice MEJ, Krawczak FS, Labruna MB, Paddock CD, Karpathy SE. 2016.
569 Development of a *Rickettsia bellii*-specific taq man assay targeting the citrate synthase
570 gene. Journal of Medical Entomology **53**(6):1492-1495.
571 Kato CY, Chung IH, Robinson LK, Austin AL, Dasch GA, Massunga RF. 2013. Assessment of
572 real-time PCR assay for detection of *Rickettsia* spp. and *Rickettsia rickettsii* in banked
573 clinical samples. Journal of Clinical Microbiology **51**:314–417.
574 Kaufman L, Rousseeuw P. 1990. Finding Groups in Data: An Introduction to Cluster Analysis.
575 John Wiley & Sons, Inc.
576 Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A,
577 Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious
578 Basic: An integrated and extendable desktop software platform for the organization and
579 analysis of sequence data. Bioinformatics **28**:1647–1649.
580 Kim D, Hofstaedter CE, Zhao C, Mattei L, Tanes C, Clarke E, Lauder A, Sherrill-Mix S,

Chehoud C, Kelsen J, Conrad M, Collman RG, Baldassano R, Bushman FD, Bittinger K. 2017. Optimizing methods and dodging pitfalls in microbiome research. Microbiome **5**:52.

Lado P, Cox C, Wideman K, Hernandez A, Klompen H. 2019. Population genetics of *Dermacentor variabilis* Say 1821 (Ixodida: Ixodidae) in the United States Inferred from ddRAD-seq SNP Markers. Annals of the Entomological Society of America **112**:433–442.

Lado P, Qurollo B, Williams C, Junge R, Klompen H. 2018. The microbiome of *Haemaphysalis lemuris* (Acari: Ixodidae), a possible vector of pathogens of endangered lemur species in Madagascar. Ticks and Tick-borne Diseases **9**:1252–1260.

Leclerque A. 2008. Whole genome-based assessment of the taxonomic position of the arthropod pathogenic bacterium *Rickettsiella grylli*. FEMS Microbiology Letters **283**(1):117-127.

Li JS, Chu F, Reilly A, Winslow GM. 2002. Antibodies Highly Effective in SCID Mice During Infection by the Intracellular Bacterium *Ehrlichia chaffeensis* Are of Picomolar Affinity and Exhibit Preferential Epitope and Isotype Utilization. The Journal of Immunology **169**:1419–1425.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal **17**:10.

McMurdie PJ, Holmes S. 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE **8**:e61217.

Murgia MV, Bell-Sakyi L, de la Fuente J, Kurtti TJ, Makepeace BL, Mans B, McCoy KD, Munderloh U, Plantard O, Rispe C, Valle MR, Tabor A, Thangamani S, Thimmapuram J, Hill CA. 2019. Meeting the challenge of tick-borne disease control: A proposal for 1000 *Ixodes* genomes. Ticks and Tick-borne Diseases **10**:213–218.

Narasimhan S, Fikrig E. 2015. Tick microbiome: the force within. Trends in parasitology

31:315–23.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2019. vegan: Community Ecology Package. R package version 2.5-2.

Paddock CD, Denison AM, Dryden MW, Noden BH, Lash RR, Abdelghani SS, Evans AE, Kelly AR, Hecht JA, Karpathy SE, Ganta RR, Little SE. 2015. High prevalence of "Candidatus *Rickettsia andeanae"*; and apparent exclusion of *Rickettsia parkeri* in adult *Amblyomma maculatum* (Acari: Ixodidae) from Kansas and Oklahoma. Ticks and Tick-borne Diseases **6**:297–302.

Paddock CD, Lane RS, Staples JE, Labruna MB. 2016. Changing paradigms for tick-borne diseases in the Americas. Page Global Health Impacts of Vector-Borne Diseases: Workshop Summary.

Price MN, Dehal PS, Arkin AP. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. Molecular Biology and Evolution **26**:1641–1650.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Research **41**:D590-D596.

Regnery RL, Spruill CL, Plikaytis BD. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. Journal of Bacteriology **173**:1576–1589.

Rynkiewicz EC, Hemmerich C, Rusch DB, Fuqua C, Clay K. 2015. Concordance of bacterial communities of two tick species and blood of their shared rodent host. Molecular Ecology **24**:2566–79.

627 Smith CCR, Snowberg LK, Gregory Caporaso J, Knight R, Bolnick DI. 2015a. Dietary input of
628 microbes and host genetic variation shape among-population differences in stickleback gut
629 microbiota. ISME Journal **9**:2515–2526.
630 Smith TA, Driscoll T, Gillespie JJ, Raghavan R. 2015b. A Coxiella-like endosymbiontis a
631 potential vitamin source for the lone star tick. Genome Biology and Evolution **7**(3):831-838.
632 Spor A, Koren O, Ley R. 2011. Unravelling the effects of the environment and host genotype on
633 the gut microbiome. Nature Reviews Microbiology **9**(4):279-290.
634 Steury RA, Currey MC, Cresko WA, Bohannan BJM. 2019. Population genetic divergence and
635 environment influence the gut microbiome in oregon threespine stickleback. Genes
636 **10**(7):484.
637 Travanty NV, Ponnusamy L, Kakumanu ML, Nicholson WL, Apperson CS. 2019. Diversity and
638 structure of the bacterial microbiome of the American dog tick, *Dermacentor variabilis*, is
639 dominated by the endosymbiont *Francisella*. Symbiosis **79**:239-250.
640 Trout Fryxell RT, DeBruyn JM. 2016. The microbiome of *Ehrlichia*-infected and uninfected
641 lone star ticks (*Amblyomma americanum*). PLoS ONE **11**:e0146651.
642 Van Treuren W, Ponnusamy L, Brinkerhoff RJ, Gonzalez A, Parobek CM, Juliano JJ, Andreadis
643 TG, Falco RC, Beati Ziegler L, Hathaway N, Keeler C, Emch M, Bailey JA, Roe RM,
644 Apperson CS, Knight R, Meshnick SR. 2015. Variation in the Microbiota of *Ixodes* Ticks
645 with Regard to Geography, Species, and Sex. Applied and Environmental Microbiology
646 **81**(18):6200–6209.
647 Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA,
648 Jansson JK, Caporaso JG, Fuhrman JA, Apprill A, Knight R. 2016. Improved Bacterial 16S
649 rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers

650 for Microbial Community Surveys. mSystems **1**(1):e0009-15.

651 Zhong J, Jasinskas A, Barbour AG. 2007. Antibiotic treatment of the tick vector *Amblyomma*

652 *americanum* reduced reproductive fitness. PLoS ONE **2**(5):e405.

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Table 1(on next page)

General information of *Dermacentor variabilis* samples used in this study. All individuals are from the USA. Each row corresponds to an individual tick specimen and the columns to the collection information.

Table 1. General information of *Dermacentor variabilis* samples used in this study. All individuals are from the USA. Each row corresponds to an individual tick specimen and the columns to the collection information.

RAW DATA ID	VOUCHER ID	COLLECTION EVENT ID	SEX	US STATE	LOCALITY	COORDINATES
119592-6	OSAL119943	OSAL119592	Female	IN	Hoosier National Forest	38.52, -86.44
119592-7	OSAL119944	OSAL119592	Male	IN	Hoosier National Forest	38.52, -86.44
119273-2	OSAL119553	OSAL119273	Female	IN	Splinter Ridge Wildlife area	38.75, -85.20
119241-2	OSAL119260	OSAL119241	Female	OH	Battelle Darby Creek MP	39.9, -83.21
119241-3	OSAL119261	OSAL119241	Male	OH	Battelle Darby Creek MP	39.9, -83.21
119241-4	OSAL119262	OSAL119241	Male	OH	Battelle Darby Creek MP	39.9, -83.21
119243-1	OSAL119267	OSAL119243	Female	OH	Glacier Ridge MP	40.13, -83.18
119243-2	OSAL119268	OSAL119243	Female	OH	Glacier Ridge MP	40.13, -83.18
119243-3	OSAL119269	OSAL119243	Male	OH	Glacier Ridge MP	40.13, -83.18
119243-4	OSAL119270	OSAL119243	Male	OH	Glacier Ridge MP	40.13, -83.18
119244-1	OSAL119400	OSAL119244	Female	OH	High banks MP	40.15, -83.03
119244-4	OSAL119403	OSAL119244	Male	OH	High banks MP	40.15, -83.03
119247-1	OSAL119404	OSAL119247	Female	OH	Pickerington Ponds MP	39.88, -82.79
119247-4	OSAL119407	OSAL119247	Male	OH	Pickerington Ponds MP	39.88, -82.79
119248-1	OSAL119392	OSAL119248	Female	OH	Pickerington Ponds MP	39.88, -82.80
119248-4	OSAL119395	OSAL119248	Male	OH	Pickerington Ponds MP	39.88, -82.80
119250-2	OSAL119397	OSAL119250	Female	OH	Sharon Woods MP	40.11, -82.95
119250-4	OSAL119399	OSAL119250	Male	OH	Sharon Woods MP	40.11, -82.95
110559-1	OSAL110503	OSAL110559	Female	OH	Fernald Preserve	39.29, -84.69
110559-2	OSAL110504	OSAL110559	Female	OH	Fernald Preserve	39.29, -84.69
119600B	OSAL129714	OSAL119600	Male	OH	Roads intersection	39.13, -84.79
115093A	OSAL129721	OSAL115093	Female	OH	Cuyahoga Valley	41.289, -81.573
115093B	OSAL129722	OSAL115093	Female	OH	Cuyahoga Valley	41.289, -81.573
115093C	OSAL129723	OSAL115093	Female	OH	Cuyahoga Valley	41.289, -81.573
115093D	OSAL129724	OSAL115093	Male	OH	Cuyahoga Valley	41.289, -81.573
115093E	OSAL129725	OSAL115093	Male	OH	Cuyahoga Valley	41.289, -81.573
119928A	OSAL129691	OSAL119928	Female	OH	Strouds Run SP	39.369, -82.042
119928B	OSAL129692	OSAL119928	Female	OH	Strouds Run SP	39.369, -82.042
119928C	OSAL129693	OSAL119928	Male	OH	Strouds Run SP	39.369, -82.042
119928D	OSAL129694	OSAL119928	Male	OH	Strouds Run SP	39.369, -82.042

119572A	OSAL129695	OSAL119572	Female	OH	Oak Openings MP	41.549, -83.854
119572B	OSAL129696	OSAL119572	Female	OH	Oak Openings MP	41.549, -83.854
119572C	OSAL129697	OSAL119572	Male	OH	Oak Openings MP	41.549, -83.854
119572D	OSAL129698	OSAL119572	Male	OH	Oak Openings MP	41.549, -83.854
119567A	OSAL129707	OSAL119567	Female	TN	Knoxville	35.390, -84.226
119567B	OSAL129708	OSAL119567	Female	TN	Knoxville	35.390, -84.226
119567C	OSAL129709	OSAL119567	Female	TN	Knoxville	35.390, -84.226
119567D	OSAL129710	OSAL119567	Male	TN	Knoxville	35.390, -84.226
119567E	OSAL129712	OSAL119567	Male	TN	Knoxville	35.390, -84.226
N8805A	OSAL129702	USNMENT01358805	Male	OK	Washita Co.	35.411, -99.059
N8805B	OSAL129703	USNMENT01358805	Male	OK	Washita Co.	35.411, -99.059
N8805C	OSAL129704	USNMENT01358805	Male	OK	Washita Co.	35.411, -99.059
N8806A	OSAL129705	USNMENT01358806	Female	OK	Washita Co.	35.411, -99.059
N8806B	OSAL129706	USNMENT01358806	Female	OK	Washita Co.	35.411, -99.059
N8464B	OSAL129701	USNMENT01358464	Male	VA	Warren Co.	38.893, -78.14
119951	OSAL129711	OSAL119951	Female	GA	Statesboro	32.42, -81.77
119952A	OSAL129715	OSAL119952	Female	GA	Statesboro	32.42, -81.77
115086	OSAL129836	OSAL115086	Male	MN	Carlos Avery	45.287, -93.122
115087A	OSAL129717	OSAL115087	Female	MN	Camp Ripley	46.076, -94.349
115087B	OSAL129718	OSAL115087	Male	MN	Camp Ripley	46.076, -94.349
119918A	OSAL129829	OSAL119918	Female	MN	Columbus	45.31, -93.02
119918B	OSAL129830	OSAL119918	Female	MN	Stutsman Co.	47.23, -98.87
N128168B	OSAL129979	USNMENT01358520	Male	ND	Stutsman Co.	47.23, -98.87
115139A	OSAL129834	OSAL115139	Female	ME	Crescent Beach	43.56, -70.23
115139B	OSAL129835	OSAL115139	Male	ME	Crescent Beach	43.56, -70.23
115140	OSAL129833	OSAL115140	Female	ME	Unknown	Unknown
119276-2	OSAL129559	OSAL119276	Female	MD	Aberdeen Providing Ground	39.46, -76.12
119276-3	OSAL129560	OSAL119276	Female	MD	Aberdeen Providing Ground	39.46, -76.12
119276-5	OSAL129562	OSAL119276	Female	MD	Aberdeen Providing Ground	39.46, -76.12
119276-6	OSAL129563	OSAL119276	Female	MD	Aberdeen Providing Ground	39.46, -76.12
115101	OSAL129845	OSAL115101	Male	CA	Napa Co	38.215, -122.33
115102A	OSAL129846	OSAL115102	Male	CA	Lake Co.	39.139, -122.886
115102C	OSAL129848	OSAL115102	Male	CA	Lake Co.	39.139, -122.886
115105B	OSAL129852	OSAL115105	Female	WA	Whitman Co.	46.623, -117.228

4
5

Table 2(on next page)

Rickettsia spp. and *Anaplasma* spp. screening results divided by state.

1 **Table 2.** *Rickettsia* spp. and *Anaplasma* spp. screening results divided by state.

State	Year of collection	Number of ticks positive for rickettsiales	Total number of ticks positive			
			R. rhipicephali	*R. montanensis*	*R. bellii*	*Anaplasma* spp.
California	2017	2/3 (66.7%)			+ (2)	
Georgia	2013, 2016	1/2 (50%)		+ (1)		
Indiana	2017	0/3				
Maryland	2017	0/4				
Maine	2017	0/3				
Minnesota	2017	0/5				
North Dakota	2017	1/1 (100%)		+ (1)		
Ohio	2016, 2017	1/31 (3.2%)		+ (1)		
Oklahoma	2017	3/5 (60%)	+ (2)			+ (1)
Tennessee	2017	3/5 (60%)		+ (3)		
Virginia	2017	0/1				
Washington	2017	0/1				
Total		11/64 (17.2%)	2	6	2	1

Figure 1

Map of the USA showing the sampling locations.

The size of the circles reflects the number of specimens analyzed from each locality, from 1 to 5.

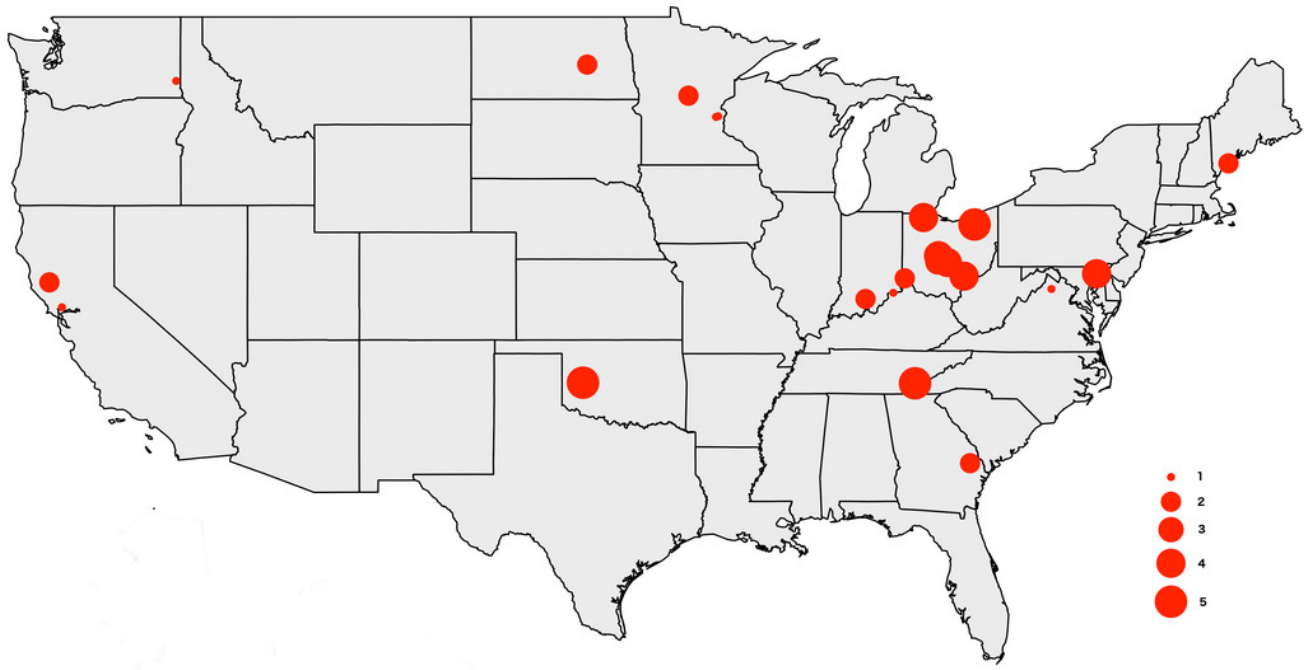


Figure 2

Barplots of the relative abundance of bacteria.

'(A) Barplot of the relative abundance of bacteria at the Order level. The x axis corresponds to individual tick specimens, and the y axis shows the relative abundance of the different bacteria Orders. Bacteria orders are color-coded as shown below the plot; (B) Similar to A), barplot of the relative abundance of the bacteria at the genus level.'

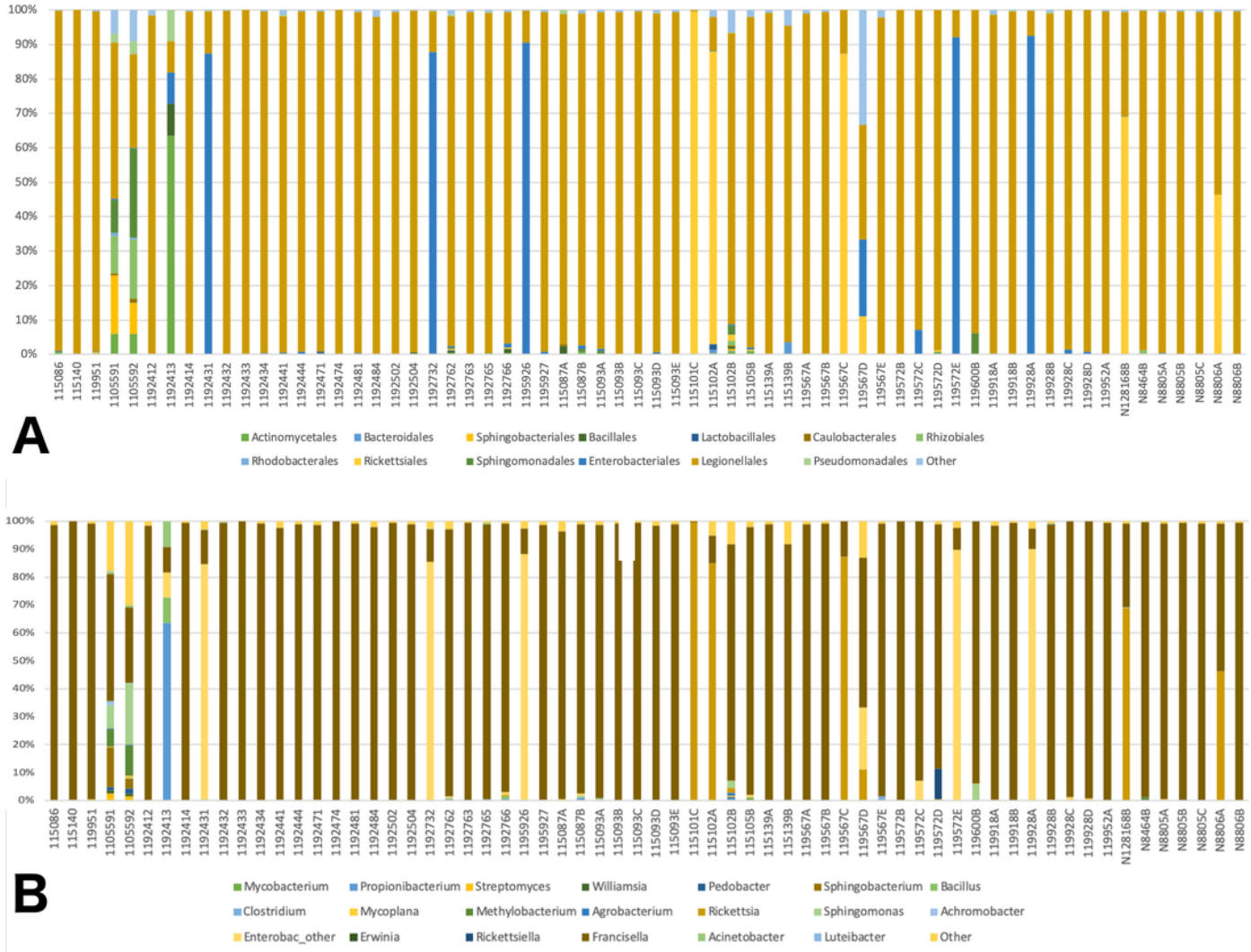


Figure 3

Dendrogram showing the relationship between all samples, and the different groupings.

Groupings names: Eastern, Western, CA (samples from California), WA (samples from Washington), and OH Hamilton Co (samples collected in Hamilton County, Ohio).

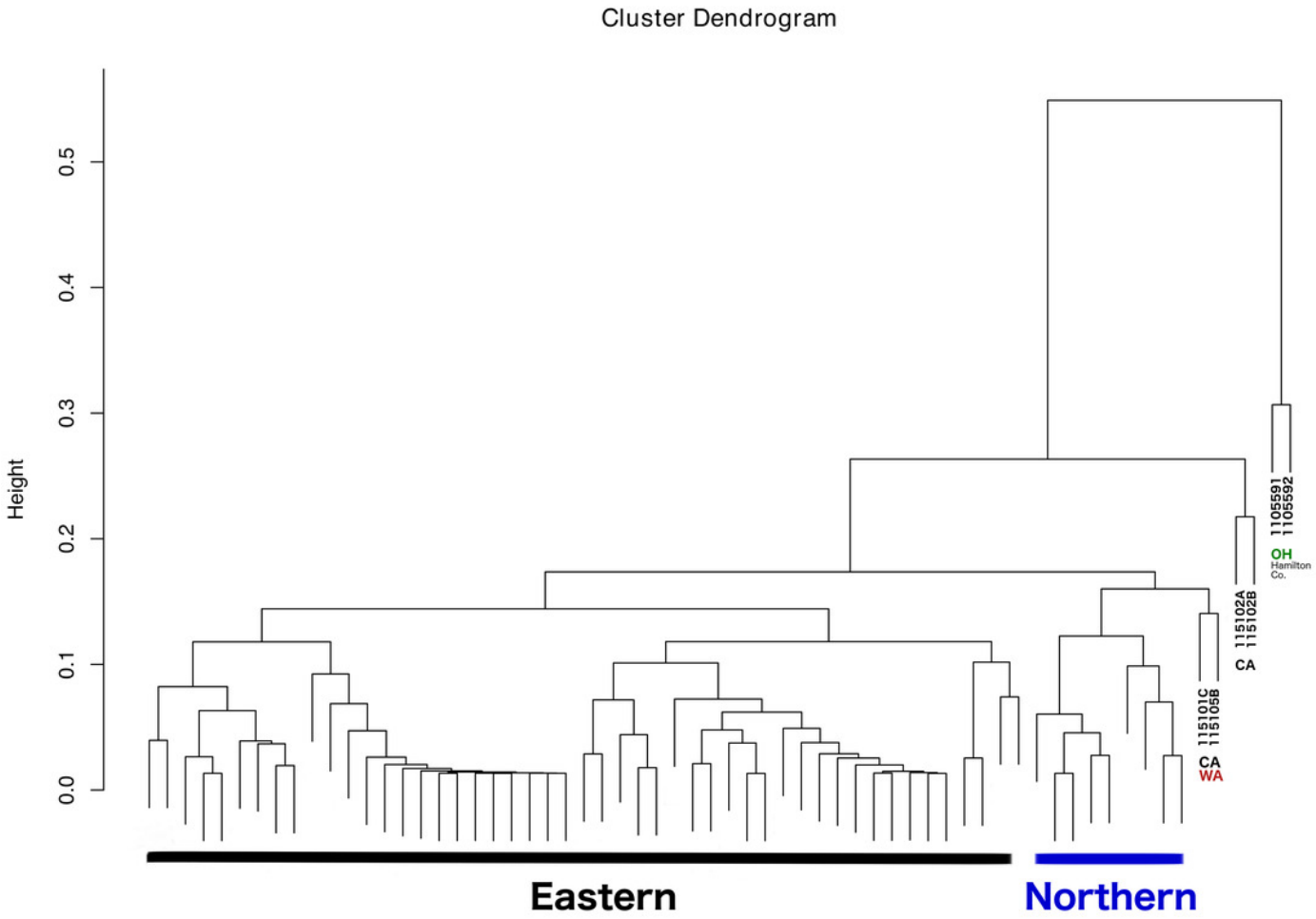


Figure 4

Non-metric multidimensional scaling and Principal coordinates analyses.

'(A) Non metric multidimensional scaling analysis of the samples. Different colors represent the different genetic clusters: Eastern (red), Northern (green), and Western (blue). (B) Principal coordinates analysis of the samples. Different colors represent the different genetic clusters: Eastern (blue), Northern (orange), and Western (black). Axis 1 explains 74.9% of the variance, and Axis 2 explains 10% of it.'

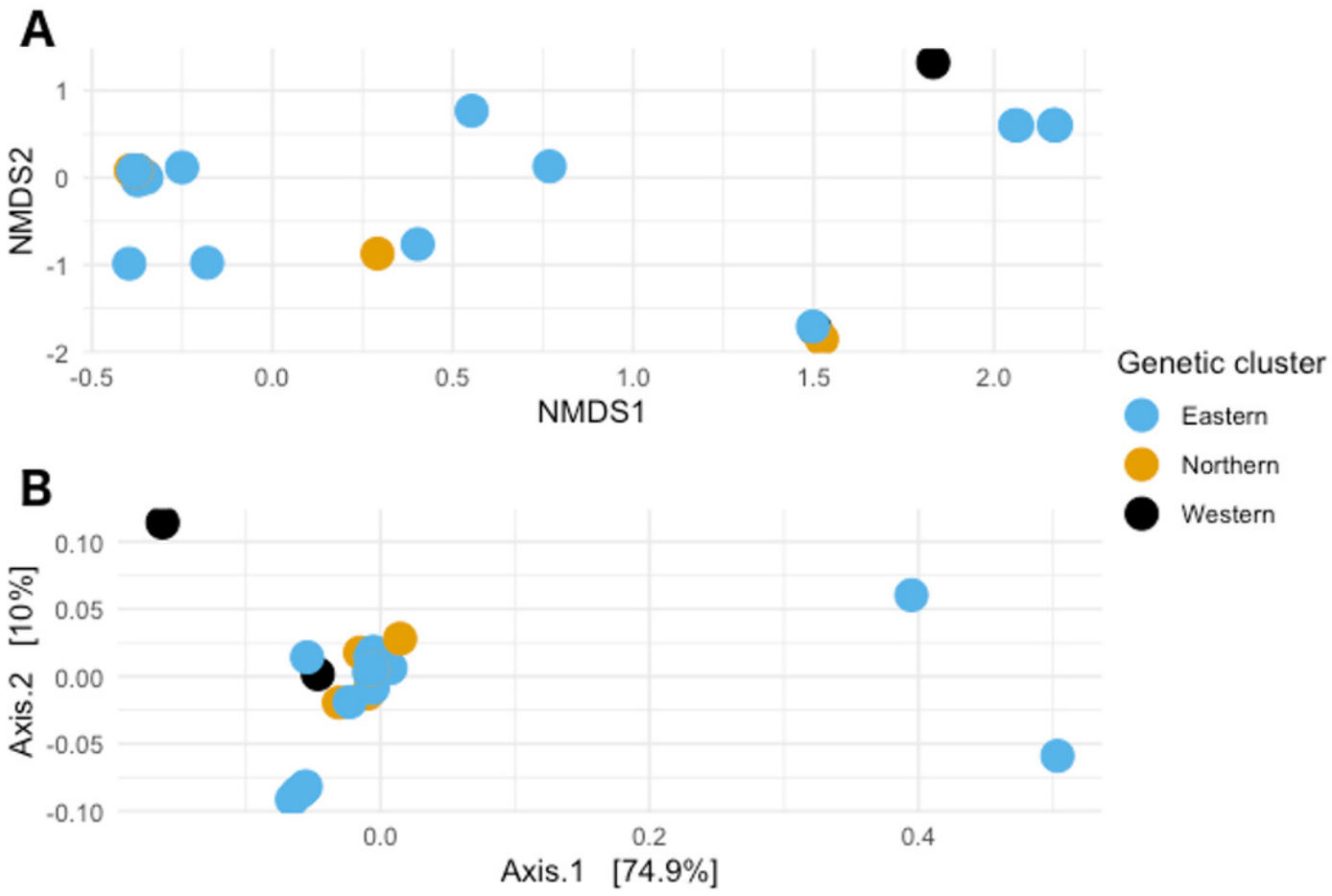


Figure 5

Average abundance of bacteria genera by genetic cluster.

Average abundance (y axis) of bacteria genera (x axis) by genetic clusters (Northern, Eastern, Western), and sex (male in orange, female in blue).

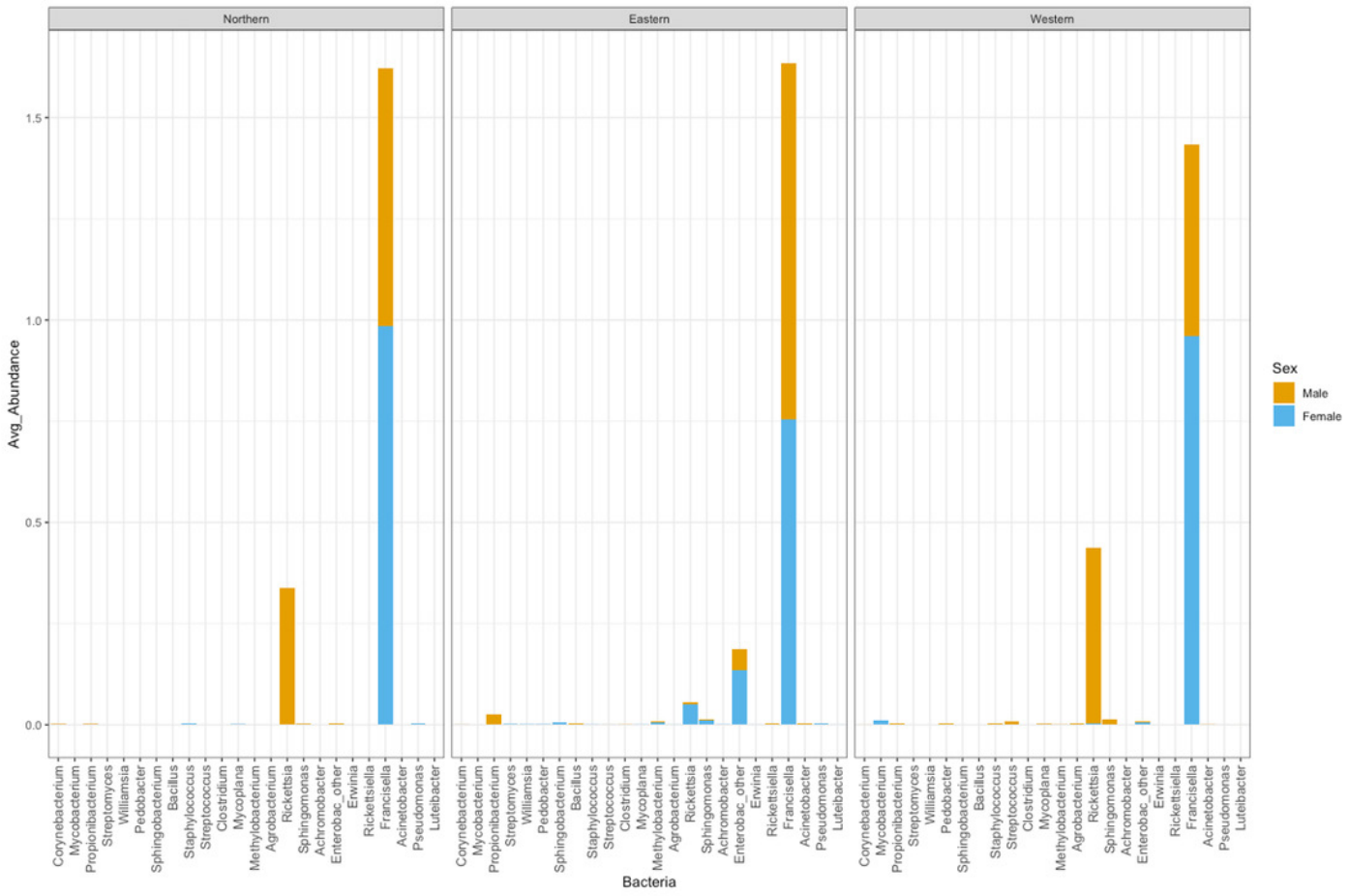
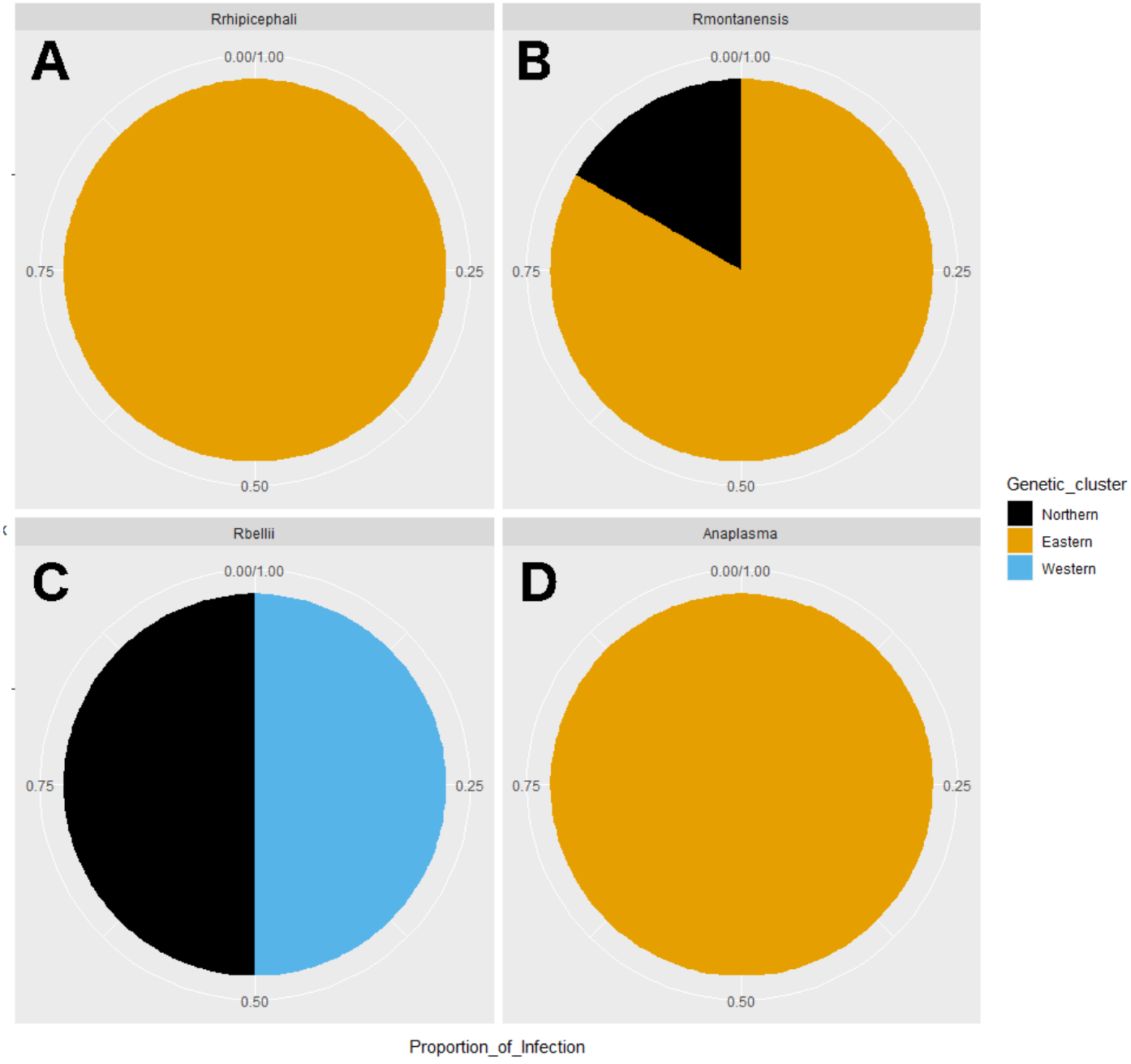


Figure 6

Pie chart plots of proportions of infection by genetic clusters.

Pie chart plots of proportions of infection by genetic clusters. Each plot corresponds to an agent; A) *R. rhipicephali*, B) *R. montanensis*, C) *R. bellii*, D) *Anaplasma* spp. The colors represent the genetic clusters (Northern in black; Eastern in orange; and Western in blue).


Rrhipicephali
A
Rmontanensis
B
Rbellii
C
Anaplasma
D
0.00/1.00
0.25
0.50
0.75
Genetic_cluster
Northern
Eastern
Western
Proportion_of_Infection