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 associations between native microbes and pathogens may provide a new promising path towards

the disruption of pathogen transmission (Narasimhan & Fikrig 2015; Bonnet et al. 2017). In the case of ticks, microbes can be acquired from the environment (e.g through the spiracles), horizontally (from the host, during blood feeding), or vertically (maternally inherited). Relationships between the tick and a particular microbe or set of microbes can range from mutualistic to parasitic (Casadevall et al. 2011; Bonnet et al. 2017). Microbes can affect tick fitness, vector competence, and pathogen transmission (Bonnet et al. 2017; Budachetri et al. 2018). For example, a few maternally inherited microbes have been proposed as primary symbionts necessary for tick success by providing vitamins that are lacking in blood (Smith et al. 2015b; Duron et al. 2017; Guizzo et al. 2017). Without these microbes, the ticks' fitness is negatively impacted (Zhong et al. 2007). Other symbionts interact with pathogenic microorganisms, either positively or negatively. High proportions of the endosymbiotic *Rickettsia bellii* inhibit the transmission of the pathogenic *Anaplasma marginale* (Gall et al. 2016), and *R. parkeri* is likely excluded from *A. maculatum* by Candidatus *Rickettsia andeanae* (Paddock et al. 2015), presumably through a phenomenon known as interference. On the other hand, cases of facilitation have also been reported; such as a positive relationship between the proportion of the microbiome occupied by *Francisella* endosymbiont (FLE) and the infection level of *F. novicida* (Gall et al., 2016).

Even though a considerable amount of information and knowledge has been generated during the last decade or so, microbiome research on ticks is still in its infancy. For example, we still do not fully understand how host genetics and environmental factors interact to shape the microbiome of organisms (Spor et al. 2011; Goodrich et al. 2014; Steury et al. 2019), or how 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

The tick specimens employed in this study are the same individual ticks from Lado et al. (2019), with the exception of two specimens for which we did not have enough genetic material. The sample includes 64 adult *D. variabilis* collected from California (n=3), Georgia (n=2),

Indiana (n=3), Maryland (n=4), Maine (n=3), Minnesota (n=5), North Dakota (n=1), Ohio (n=31), Oklahoma (n=5), Tennessee (n=5), Virginia (n=1), and Washington (n=1) (Fig. 1). All specimens were wild caught by dragging a 1m x 0.8m cloth thorugh the vegetation, and preserved in 95% ethanol. We collected ticks from vegetation (unfed) to minimize the amount of genetic material from the host. Collection data for the specimens used in this study can be accessed through the Ohio State Acarology Collection (OSAL) online database (https://acarology.osu.edu/database). Metro Parks permitted collection of tick species throughout the Metro Parks. The Division of State Parks (DNR), Indiana Department of Natural Resources authorized tick collection at Brown County State Park, Clifty Falls State Park, O'Bannon Woods State Park, Monroe Lake, Splinter Ridge Fish and Wildlife Area, Clark State Forest, and Ferdinand State Forest.

DNA extraction

Before DNA extraction the ticks were surface sterilized following Lado et al. (2018); specifically, the washes consist on following this procedure twice: commercial bleach 3% for one minute, washed in distilled water, and 95% ethanol for another minute. DNA extractions of individual tick specimens were performed using the QIAgen Blood and Tissue kit following the manufacturers' instructions, with one exception as in Lado et al. (2018): during the incubation in ATL buffer the posterior-lateral part of the tick idiosoma was cut with a scalpel to allow a better penetration to the buffer to the tick's tissues. The cuticle of all ticks was recovered and kept as voucher. OSAL accession numbers are listed in Table 1. Genomic DNA was quantified using a Qubit 3.0 fluorometer; and then aliquoted and kept in the freezer until used.

Population genetic data

144 For the integrative portion of the analysis, we employed all genetic data generated
145 previously, and followed the genetic clusters nomenclature from Lado et al. (2019). Details on
146 how the population genetics data set was generated and analyzed can be found elsewhere (Lado
147 et al. 2019). In brief, ticks were assigned to three different genetic clusters according to their
148 population genetic structure: a generally "western cluster", an "eastern cluster", and a "northern
149 cluster". This clusters usually correspond to the locations where ticks were collected.
150

151 **Detection of Rickettsiales through PCR**

152 PCR screening

153 Tick DNAs were tested by real-time PCR to detect three genera that include pathogenic
154 microorganisms: *Rickettsia*, *Anaplasma*, and *Ehrlichia.* All ticks were initially screened using
155 two real-time assays: 1) a TaqMan Pan-*Rickettsia* assay, which amplifies a portion of the 23S
156 gene for all *Rickettsia* species using primers PanR8-F and PanR8-R (Kato et al. 2013), and 2) an
157 EvaGreen Anaplasmataceae assay that targets a portion of the 16S gene using primers
158 ECHSYBR-F and ECHSYBR-R, amplifying all *Anaplasma* and *Ehrlichia* species (Li et al.
159 2002).

160 Positive tick samples for Pan-*Rickettsia* (23S real-time assay) where further screened to
161 identify the *Rickettsia* species present. Samples were subjected to conventional semi-nested PCR
162 targeting the *omp*A gene of all spotted fever group *Rickettsia* (Regnery et al. 1991; Eremeeva et
163 al. 2006). PCRs were performed using 1 μM of each primer (Rr190.70, Rr190.602, Rr190.701),
164 10 μL of Taq PCR Master Mix (QIAGEN), 2 μL of sample DNA in the primary reaction or 2 μl
165 of the primary reaction product in the secondary reaction, and water to bring the final reaction
166 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 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 in 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 was successfully generated for all ticks. The number of Illumina forward (R1) reads after quality filtering was 2,129,331 with and average length of 251 bp. After OTU picking and taxonomic assignment many of the reads were categorized as "unassigned" and therefore, the average number of sequences per tick specimen included in downstream analyses was fairly low, ranging from a few hundred to ~15,500.

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, 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 0.5 to 100% (mean = 80.4%, median = 99.1%). *Rickettsia* was detected in 10 (15.6%) 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

282 results are overall, congruent (Table 2). *Rickettsiella*, an intracellular endosymbiont of
283 arthropods (Leclerque 2008), was found in a tick from Ohio (1/64; tick ID 119572D) with a
284 relative abundance of 11%.

285 **Statistical analysis: integrating individual data sets**

286 Clustering approach

287 The dendrogram resulting from hierarchical clustering showed that the two most distinct
288 samples (clustering together) were from the same location and collection event: Fernald
289 Preserve, Hamilton Co., Ohio (110559-1 and 110559-2) (Fig. 3). The second clade grouped two
290 samples from CA, the two samples collected in Lake Co (115102A,B). The next pair of samples
291 corresponded to the two remaining samples from the west coast, a sample from Napa Co, CA
292 (115101C); and one from Whitman Co, WA (115105B). These two samples were closely
293 associated with a clade that included all Northern samples. Lastly, all Eastern samples clustered
294 together, without any internal grouping according to geography (Fig. 3).
295 No differences were evident upon visualization of community membership or structure. PCA did
296 not show any specific clustering pattern between samples (Supplementary file 1). In fact, most of
297 the ticks were clustered in a single aggregate. The ticks that clustered somewhat separated were
298 firstly the two that also appeared separated in the clustering analysis (110559-1 and 110559-2);
299 and a tick from Ohio, 119241-3. It is important to note that the first two principal components
300 explained only approximately 40% of the variance present in our sample.

301 As for k , the optimal number of clusters, silhouette method and elbow method suggested
302 $k = 2$ and $k = 4$ respectively (Supplementary file 2). Both values were close to the number of
303 genetic clusters, which was also the number of clusters we used in the analysis. The C-index
304 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 samples were spread out without any specific pattern (Fig. 4A). In PCoA with Gower's distance as metric, most samples appeared in a single tight aggregate, while a few others dispersed randomly along the axes (Fig. 4B).

Test for significance for different variables

The number of bacterial genera found in ticks belonging to the Eastern genetic cluster (24) was higher than that of the Western (16), and Northern (10) clusters. The genus *Francisella* was dominant in almost all the ticks, regardless of geography. Its relative abundance did not differ significantly between males and females (p-val 0.8); nor between ticks belonging to different genetic clusters (Eastern vs Western p-val 0.57, Eastern vs Northern p-val 0.96, Northern vs Western p-val 0.60) (Fig. 5). The relative abundance of this genus was also not significantly different between infected and uninfected ticks (p-val 0.14), although it was generally lower in infected ticks. Infected ticks were defined as those that were PCR positive for either *Anaplasma* or *Rickettsia* species.

Rickettsia relative abundance did not significantly differ between males and females (p-val 0.44), nor between ticks belonging to different genetic clusters (Northern vs. Eastern p-val = 0.26; Northern vs. Western p-val = 0.72; and Eastern vs. Western p-val = 0.45) (Fig. 5). *Rickettsia rhipicephali* and *Anaplasma* spp. were present only in ticks belonging to the Eastern genetic cluster, and *R. montanensis* was present in ticks belonging to the Northern and Eastern clusters. *Rickettsia bellii* was found infecting ticks from the Western and Northern genetic clusters, although of these ticks were collected in the Western region (Fig 6). One of the ticks

positive for this species was 115101C, a tick collected in California but with the genetic make-up corresponding to Northern ticks.

Overall, microbial beta diversity differed between regions (p-val <0.05), but not between sexes (p = 0.63) when using Bray-Curtis distance. Post hoc tests indicated that ticks collected in the Western region harbored a different microbiome from those collected in the Eastern or Northern regions ($p < 0.05$). In the case of presence/absence of Bacteria (Jaccard distance), the results were the same. A significant difference between ticks collected from different regions was detected ($p< 0.05$), and the post hoc tests showed that the differences were significant between the Western and both the Eastern and Northern regions.

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

DISCUSSION

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

We developed an effective pre-processing and processing procedure for researchers interested in microbiomes of small organisms, or parts of organisms with limited amounts of genetic material. This was motivated by a need for consensus regarding the pre-processing of 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, together with the determination of whether or not they are capable of causing disease in humans should be further explored. Furthermore, due to the finding of this agent's DNA in a *D. variabilis* sample in this study, the role of *D. variabilis* as potential vector should be considered and further explored.

With respect to the primary goal of this study, the integration of the different types of data was done successfully, although the interpretation of the results is somewhat challenging. The latter problem is most likely caused by to the low levels of genetic diversity and moderate levels of population structure (see details in Lado et al. 2019), and a microbiome highly dominated by a few taxa. As a result, ordination methods failed to show patterns of variation across different groups. Nonetheless, the dendrogram resulting from the clustering analysis, in which all lines of evidence and geographic location were considered, was largely congruent with the observations at the population genetic level. Thus, the addition of microbiome, pathogen presence, ticks sex, and geographic location led to conclusions that are, in general, consistent with ticks' genetics. All Eastern samples clustered together (with the exception of two samples from Hamilton Co, OH), and separated from both Western and Northern samples. The distinctiveness and separation of those two samples from Hamilton Co, OH reflects their distinctiveness at the microbiome level (Fig. 2). The fact that both samples appeared very similar to all other Eastern samples at the population genetic level (Lado et al. 2019), supports

418 that hypothesis. It is possible that these two specimens were not well surface sterilized, or that
419 the environment at that collection location is different.

420 The higher number of bacterial genera found in ticks belonging to the Eastern genetic
421 cluster when compared to either Northern or Western ticks, is likely a product of the higher
422 number of ticks analyzed, and the greater geographic area covered. Even though in the broader
423 sense the microbiome is not diverse (dominated by three genera), it differed between the three
424 main geographic regions: Eastern, Western, and Northern. Statistical results looking at the beta
425 diversity between genetic clusters are less clear: initial test demonstrated a difference in the
426 microbial communities between genetic clusters, but pairwise tests with their corresponding
427 corrections failed to reach the same result. This may be a result of one of the study's limitations,
428 the number of samples. Thus, these findings should be further explored using more samples, and
429 more samples per genetic cluster. From our analyses, geography explains the differences in the
430 microbial communities better than host genetics. This could be the result of certain
431 microorganisms occurring only in certain geographic areas.

432 Despite challenges arising during the interpretation of the results, the integration of lines
433 of evidence and metadata, revealed that Eastern ticks can be separated from Northern and
434 Western ticks. It can also be noted that some *Rickettsia* species were associated with a certain
435 geographic area. For example, *R. bellii* was found only in samples collected in CA, a finding
436 consistent with that reported in Hecht et. al. (2019). It seems possible that *R. bellii* is more
437 common along the west coast, although more samples should be analyzed to confirm this. And
438 while the two *R. bellii* positive tick samples were collected in CA, one of them (ID 115101C) did
439 not belong to the Western genetic cluster; it belonged to the Northern cluster. From the host
440 genetics perspective, this tick is more similar to ticks from Northern locations; whereas from the

441 rickettsial agents perspective, it is more similar to Western samples. Looking at the microbiome
442 composition, this tick could belong to any geographic region. This underscores the value of
443 integrating different types of data when thinking about disease ecology.
444 The approaches taken during this study, both to generate and analyze data, can be applied
445 to a wide variety of taxa, and groups of organisms. It is likely that in organisms with a higher
446 level of variation, the clustering methods will be more informative, and their interpretation more
447 straightforward. Therefore, the approaches used herein have potential, and could greatly improve
448 future studies looking at different aspects of diseases ecology. In the particular case of *D.*
449 *variabilis,* the approaches used herein failed to detect clear tendencies or patterns. This was
450 likely due to the low levels of variation (both in the microbiome and population genetics), and
451 the very low prevalence of pathogenic microorganisms.
452
453 **CONCLUSIONS**
454 In conclusion, this is the first study that successfully generated microbiome, population
455 genetics, and pathogens presence data for the same individual ticks. General methodologies and
456 pre-processing steps are replicable, and applicable to different groups of organisms across the
457 tree of life. This work also comprises, at present, one of the few studies aiming at integrating
458 population genetics and microbiome data to better understand ecological processes and disease,
459 and it is the first one to do so for ticks. The integration of different lines of evidence allows a
460 more holistic approach; and clustering and ordination methods are very helpful to summarize and
461 visualize the results. Finally, this study comprises the first report of "Uncultured *Anaplasma* sp.
462 clone 15-3642" in *D. variabilis*. This agent was previously isolated from human blood, and it is

463 important to determine if it is capable of causing disease in humans, and if so, the role of
464 *Dermacentor* ticks as potential vectors.
465
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477
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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.

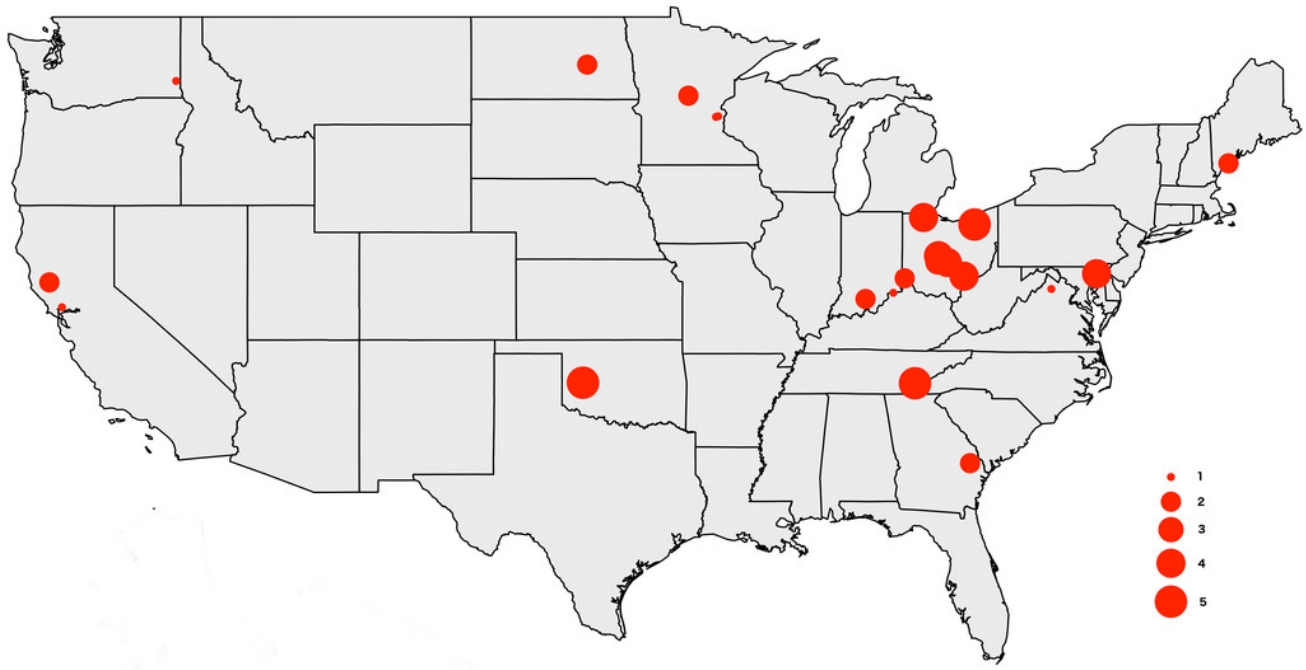


Figure 2

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.'

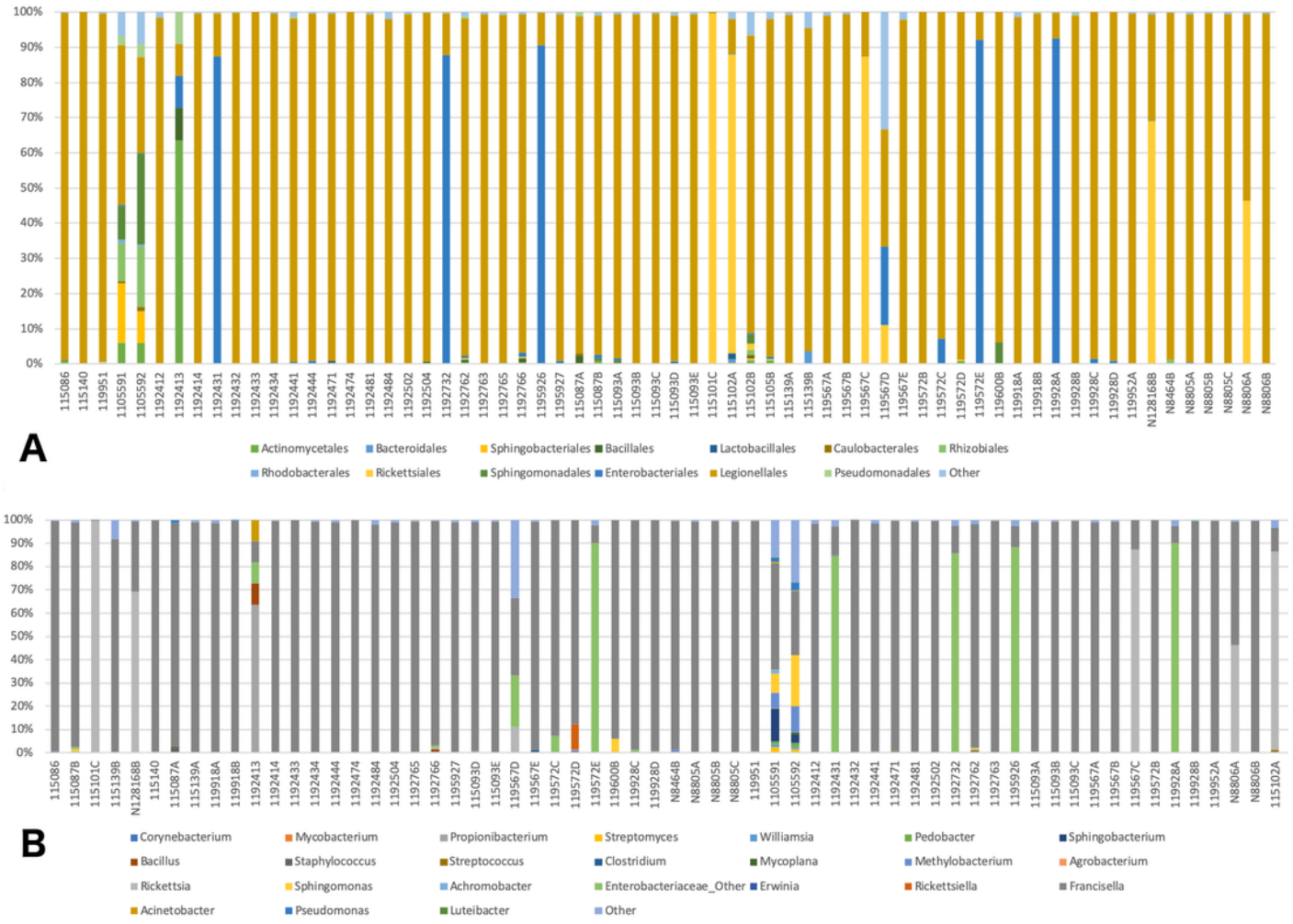


Figure 3

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).

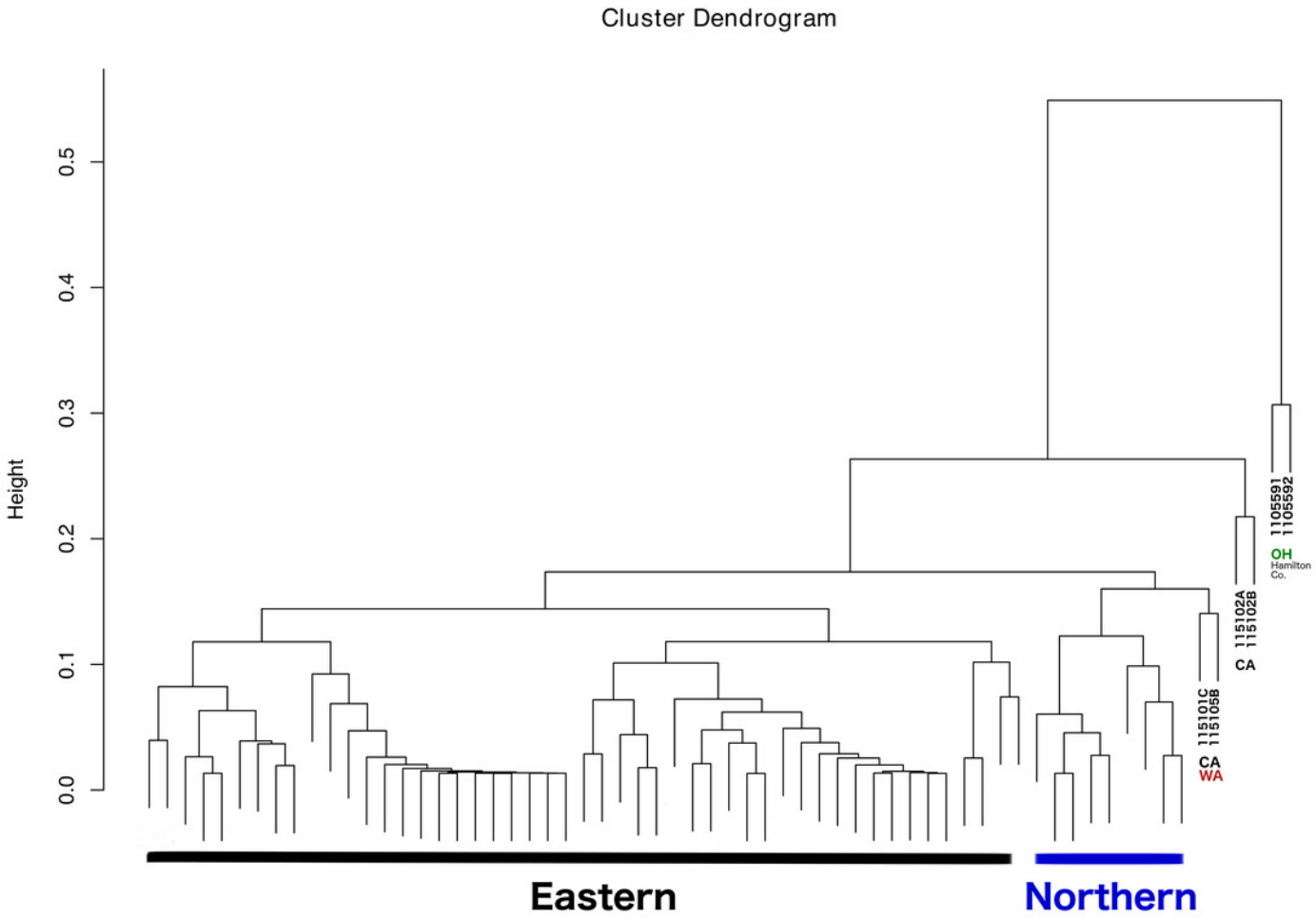


Figure 4

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.'

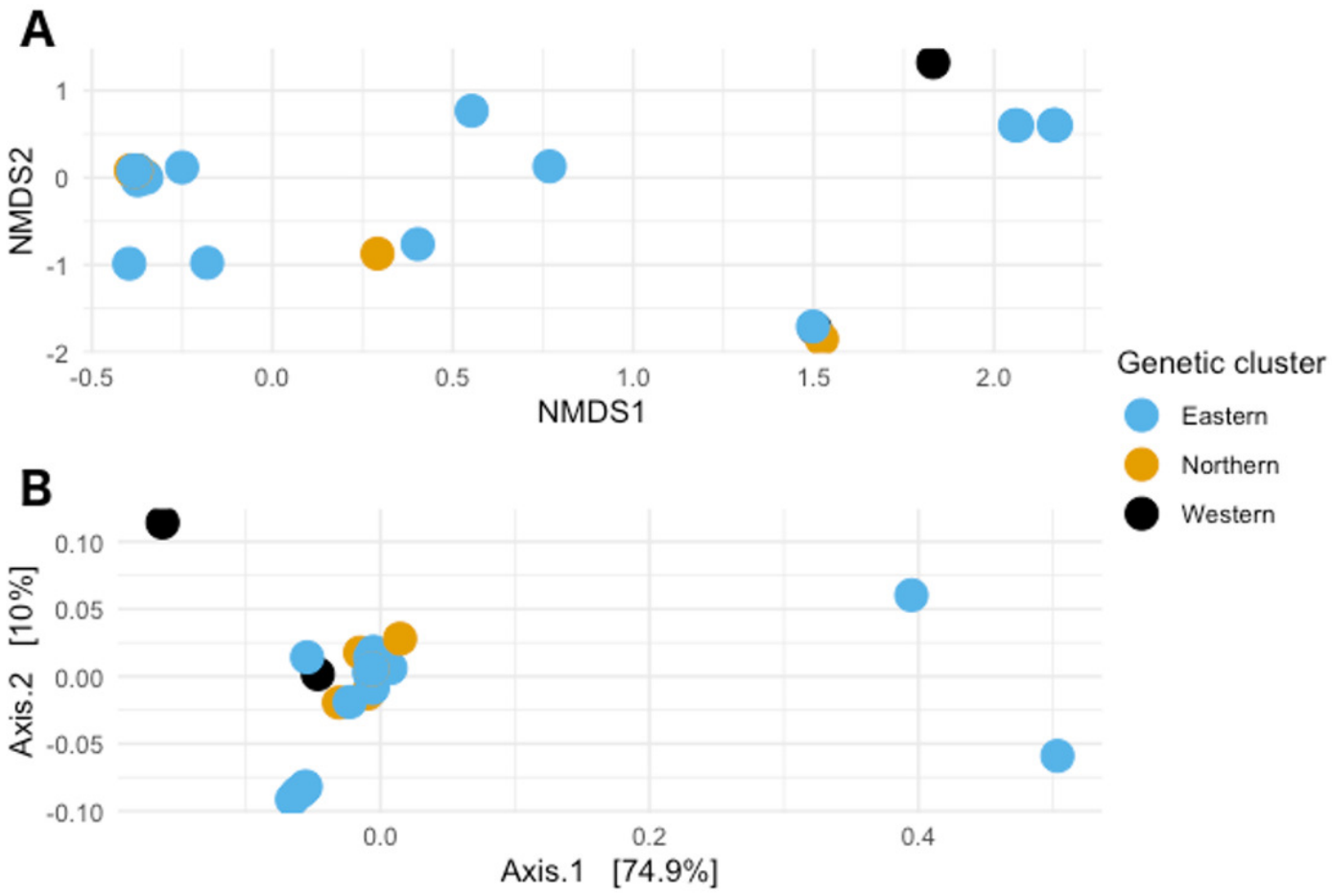


Figure 5

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 red, female in blue).

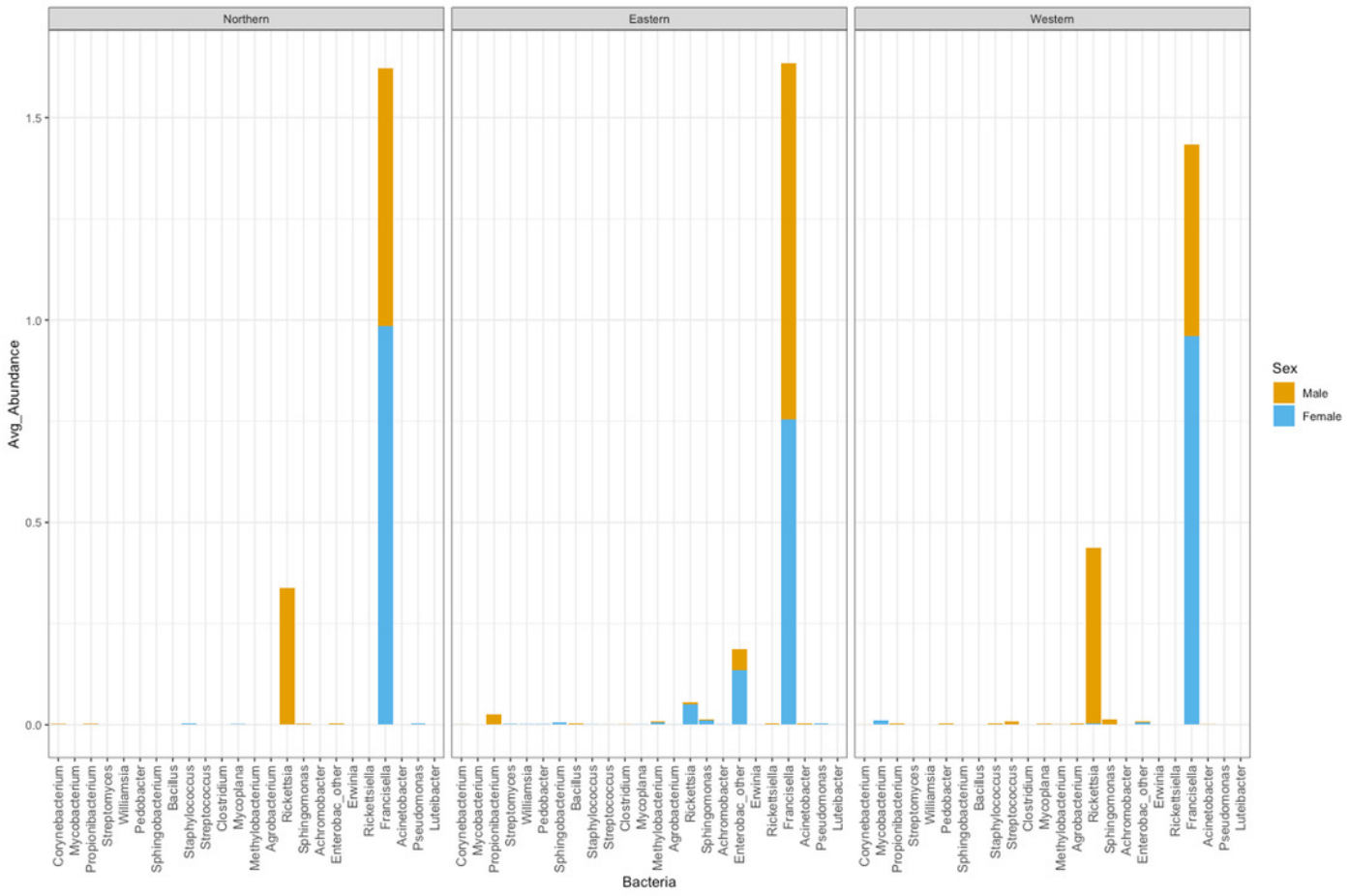
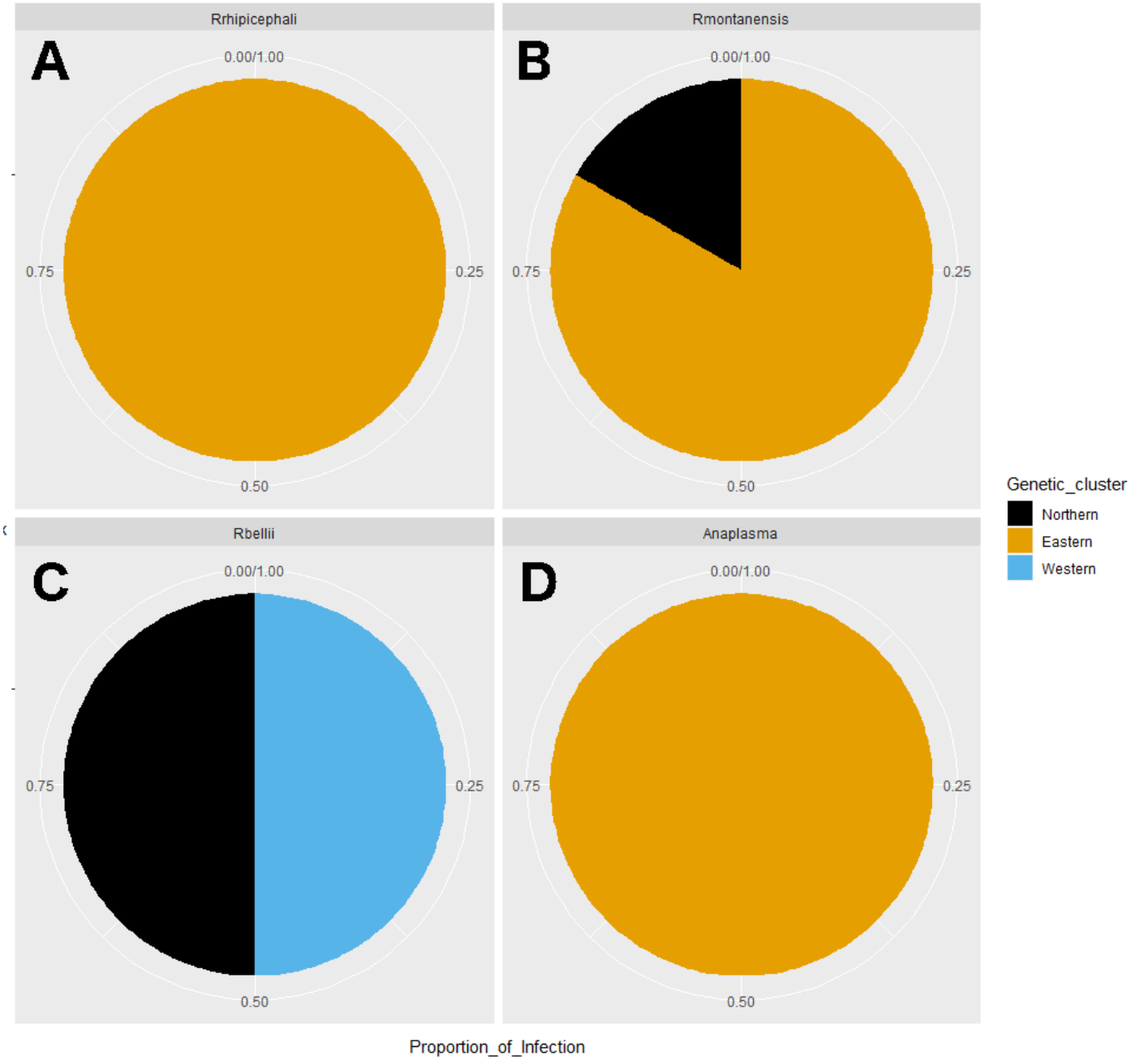


Figure 6

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