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Spatial Variation of False Map Turtle (*Graptemys pseudogeographica*) Bacterial Microbiota in the Lower Missouri River, United States

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Abstract.—**Turtle populations around the world are continually confronted with changing environments that affect their ecology and conservation status. Among freshwater turtles, population dynamics are thought to be mediated by complex yet often cryptic causes. One recent direction of focus in addressing these causes is the turtle-associated microbiota. In turtles, the gut-associated microbiota is of exceptional interest due to its continual association with host species under changing conditions. Diet-based fluctuations and changes in microbial diversity may correspond to varying external environments at both the individual and population level. Environmental responses are of particular interest due to the anthropogenic changes that may underlie them. Pollutants, disruption of climatic patterns, and habitat fragmentation all have the potential to affect turtle-associated microbiota and subsequent population and species conservation. To better understand potential human-induced changes, the diversity of turtle-associated microbiota over local spatial gradients must be better understood. We examined microbial community α- and β-diversity among 30 adult False Map Turtles (*Graptemys pseudogeographica*) at three sites within the lower Missouri River, United States. Our results indicate significant microbial community centroid differences among sites (β-diversity), which are likely mediated by various local environmental factors. Such factors will have to be carefully considered in any future attribution of anthropogenic determinants on turtle-associated microbiota as it relates to turtle population dynamics.**

Host-associated microbial communities exhibit complex interactions with their hosts and the surrounding environment. Interactions between hosts and their microbial communities have the potential to alter individual host health and development (Sommer and Backhed, 2013), and can also affect the evolution and ecology of populations and communities (Zilber-Rosenberg and Rosenberg, 2008). Host–microbiota interactions often result in complex dynamics that correlate with factors, both extrinsic and intrinsic to hosts, such as environment, diet, and disease. In aquatic organisms, the near constant contact between a changing aquatic environment and the corresponding microbial communities influences microbiota composition and function on the host gastrointestinal tract and skin (Hentschel et al., 2012).

As the gastrointestinal tract is subject to changing external environmental conditions, cloacal microbiota have received attention as a way to effectively and noninvasively study the bacteria community of the distal section of the gastrointestinal tract, especially in reptiles (Martin et al., 2010; Charruau et al., 2012; Jiang et al., 2017). In turtles, bacterial microbiota research continues to be an understudied but growing field. Microbiota of Green Sea Turtles (*Chelonia mydas*) in particular represent one of the better-studied turtle microbiota systems. Studies of Green Sea Turtle–associated bacteria communities include examination across habitat types (Price et al., 2017), different locations along the digestive tract (McDermid et al., 2020), pre- and post-hospitalization (Asahan et al., 2018), and as a function of diet (Campos et al., 2018). Additionally, the bacterial microbiota of cloacal material from Loggerhead Sea Turtles (*Caretta caretta*) has been characterized (Abdelrhman et al., 2016). Host microbiota studies among freshwater turtle species are scarcer. However, studies of microbiota associated with fecal material in Painted Turtles (*Chrysemys picta*) have been recently conducted (Fugate et al., 2020), as well as research on the effects of glyphosate on the cloacal microbiota of False Map Turtles (Madison et al., 2018). Here, we expand our work on False Map Turtles (*Graptemys pseudogeographica*) to examine the effect of host spatial distribution on the cloacal microbiota.

Graptemys pseudogeographica is a state-threatened species of riverine turtle in South Dakota, United States (Ashton and Dowd, 2008). In the state, they are primarily distributed along the Missouri River and moderate distances up associated tributaries (Ballinger et al., 2000; Davis, 2018). Prior to the creation of several large reservoirs, *G. pseudogeographica* was considered the most abundant turtle in the Missouri River (Timken, 1968); however, their declines in four large reservoirs have led to their status as a species of conservation concern. In free-flowing segments of the Missouri River, such as the 59-mile stretch of the Missouri National Recreational River from Yankton, South Dakota to Ponca, Nebraska, *G. pseudogeographica* remain abundant (Gregor and Swanson 2008). Given the growing understanding of the importance of host-associated microbiota to host health, and its potential to be influenced by environmental and host factors, it is increasingly important to understand *G. pseudogeographica*–associated microbiota composition.

Graptemys pseudogeographica microbiota is thought to be influenced by various factors including diet and range, which are largely dependent on available resources (Vogt, 1981). Individuals feed on both the water surface and underwater, exhibiting a feeding behavior known as benthic bulldozing, where the turtles will indiscriminately consume river bottom detritus (Moll, 1976). *Graptemys pseudogeographica* home range is also variable and seemingly dependent on various environmental factors. Previous work has recorded total interriver ranges as 5,152 m for females and 3,753 m for males (Bodie and Semlitsch, 2000). Additionally, female *G. pseudogeographica* have

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been recaptured up to 8 km from their previous nesting sites of the same year (Vogt, 1980). Variations in diet, feeding styles, and interriver dispersal patterns therefore present a potential for dynamic interactions between the bacterial microbiota of the cloaca and the external environment. Here, we hypothesized that spatial gradients would exhibit significant differences in community composition of *G. pseudogeographica* bacterial microbiota. To test our hypothesis, we examined *G. pseudogeographica* cloacal microbiota and related water temperature data at three locations along the lower Missouri River (Nebraska and South Dakota, United States). The results of our investigation are important for the interpretation of intraspecies turtle microbiota variation as a function of changing environmental conditions and sampling location.

Materials and Methods

Study System and Sample Collection.—We collected *G. pseudogeographica* at three sites along the 59-mile stretch of the Missouri National Recreational River between Yankton and Burbank, South Dakota, United States. Our study sites were located on a region of the Missouri River that retains some semblance of preregulation channel morphology and habitats, with riparian forests, sandbars, and islands present along the river channel, though river flow and sediment regimes remain modified due to the Gavins Point Dam upstream (Dixon et al., 2015). Outside of the immediate river boundary, much of the landscape encompassing our sites along the Missouri River and its input tributaries is characterized as intensive agricultural land use (SDDENR, 2020). Our three study localities included: 1) the confluence of the James River with the Missouri River, 2) the Missouri River surrounding Goat Island, and 3) the confluence of the Vermillion River with the Missouri River, from west (upstream) to east (downstream) respectively (Fig. 1). Turtle capture and sampling at our localities were conducted over a 28-d period during June and July of 2017. Using partially submerged hoop traps baited with sardines, we captured 30 *G. pseudogeographica*. Traps were left submerged near basking surfaces (e.g., fallen trees) for 24–48 h and captured individuals were measured (straight-line carapace length), sexed, given a unique identifying notch on their marginal scutes for future studies (following a modified version of Ernst et al. [1974]), and photographed. Next, we inserted a sterile rayon-tipped swab (No. MWE113, Medical Wire & Equipment, Corsham, Wiltshire, UK) into the cloaca, rotated fully three times, before gently removing it. After data and swab collection, we released all individuals at their site of capture. Swab samples were stored individually in sterile microcentrifuge tubes and held on ice in the dark while in the field until transported to a −20°C freezer (<4 h), where they stayed until DNA extraction and purification. Additionally, we collected water temperature data from each of the three sampling regions using a YSI meter (Xylem Inc., Yellow Springs, Ohio, USA).

DNA Extraction and Purification.—We completed DNA extractions of all samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The protocol included an overnight tissue digestion with proteinase K at 56°C. After extraction, we concentrated and purified the DNA using the ZR-96 Genomic DNA Clean & Concentrator-5 Kit (Zymo Research, Irvine, California, USA) by using the provided standard protocol.

Library Preparation and High-Throughput Sequencing.—We used all of the 30 *G. pseudogeographica* samples for high-throughput sequencing. In conjunction with the Westcore facility at Black Hills State University (BHSU, Spearfish, South Dakota, USA), we completed 16S rDNA sequencing. We quantified extracted DNA from each sample using the Qubit dsDNA HS Assay Kit (quantitation range: 0.2–100 ng) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The quantification included a modified dual-indexing protocol developed by Illumina (Illumina 16S Metagenomic Sequencing Protocol [15044223 Rev. B]), with ≤15 ng of DNA from each sample for producing a library for high-throughput sequencing. Two rounds of amplification occurred, during the first of which primers targeted the V4 region of the 16S rRNA gene with the 2× KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, Massachusetts, USA). We ran each sample in duplicate. Here, we used a thermocycling protocol as follows: initial denaturation at 95°C for 3 min, followed by 25 cycles of 98°C for 20 sec, 55°C for 15 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min on a Veriti Thermal Cycler (ThermoFisher Scientific, Waltham, Massachusetts, USA). We then added Illumina overhang adapters with modified 515F and 806R primers (V4_515F: 5′–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG [GTG YCA GCM GCC GCG GTA A]–3′ and V4_806R: 5′–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G[GG ACT ACH VGG GTW TCT AAT]–3′; 515F and 806R base primers are bracketed). We purified all reactions prior to indexing using Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA). For indexing and subsequent purifications, we used a bead solution:PCR product ratio of 0.8 (20 μL of bead solution with 25 μL of PCR product).

We completed secondary amplification using 2× KAPA HiFi HotStart Ready Mix, as well as a combination of two unique Nextera XT Index primers (N7xx and S5xx) on a Veriti Thermal Cycler. The secondary amplification protocol used followed the primary amplification cycle as given above. We then purified the libraries again with Agencourt AMPure XP beads and subsequently quantified these libraries using the Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer, normalized, and pooled together. We used the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, Wisconsin, USA) to gel-repurify the final library. We performed 300 bp paired-end sequencing on a MiSeq instrument using the MiSeq Reagent Kit v3 with the 600-cycle option (Illumina Inc., San Diego, California, USA).

Sequence Data and Statistical Analysis.—We completed our initial processing and analysis of high-throughput sequencing data using mothur (v1.39.5; Schloss et al., 2009). Briefly, we followed a standard MiSeq data processing protocol starting with generating contigs from paired-end reads, clean-up steps (including screening for low-quality reads, filtering out these reads, and chimera removal), alignment to the Silva database (v1.32), and generation of OTUs for statistical analysis (Kozich et al., 2013, accessed 29 January 2018). We calculated and visualized community abundance and α- and β-diversity metrics with the R statistical language (v3.6.2; R Core Team, 2019). R packages used included phyloseq, ggplot2, vegan, dplyr, Grid, reshape2, dunn.test, and pairwiseAdonis (Wickham, 2012, 2016; McMurdie and Holmes, 2013; Oksanen et al., 2013; Wickham et al., 2016; Dinno, 2017; Martinez Arbizu, 2020). We used nonmetric multidimensional scaling to visualize ordinal distances of community β-diversity using the Bray-Curtis distance in two-dimensional Euclidean space with a square root transformation and Wisconsin double-standardization. Permutational multivariate analysis of variance (PERMANOVA) analyses were completed using the adonis function in vegan. This was followed by pairwise multiple

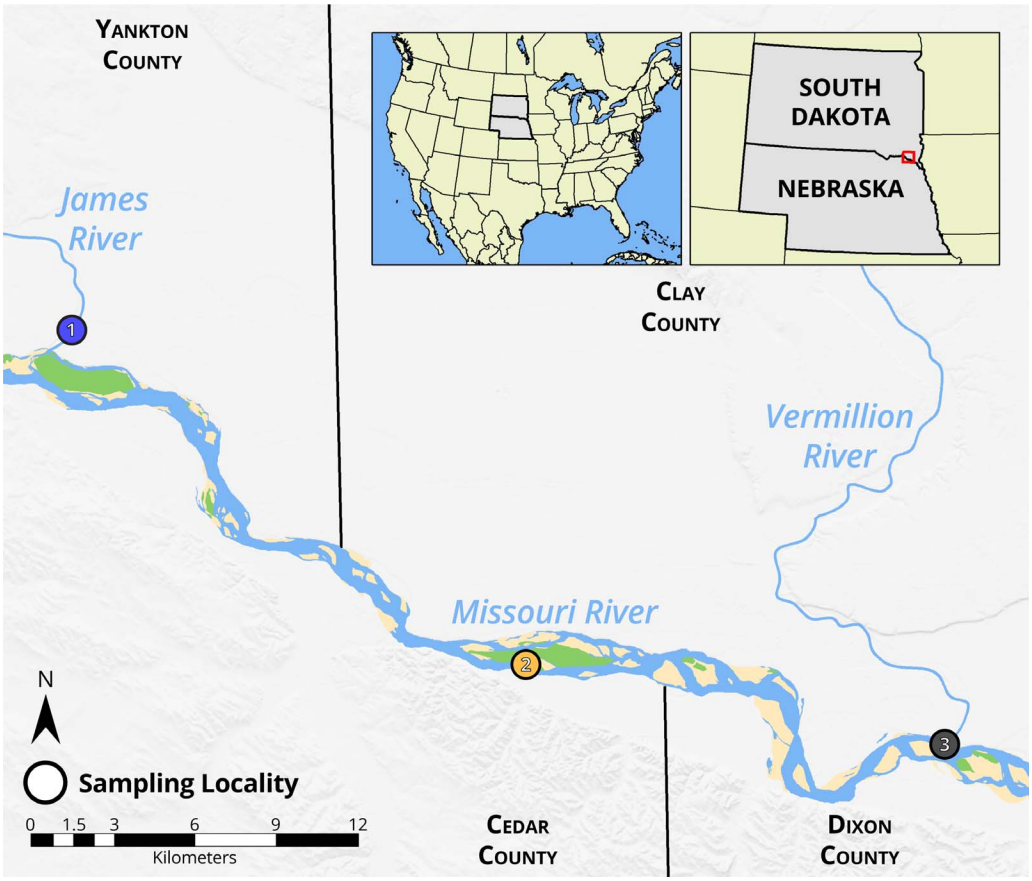

FIG. 1. Locations where cloacal microbiota samples were taken from False Map Turtles (*Graptemys pseudogeographica*) along the lower Missouri River between South Dakota and Nebraska, United States. Sites include: 1) James River, 2) Goat Island, and 3) Vermillion River. Major habitat types along the Missouri River are indicated (green = riparian forests; tan = sandbars) and modified from Dixon et al. (2015).

comparisons using the pairwiseAdonis package and β-dispersion analysis using permutational tests and Tukey's HSD where applicable. For the α-diversity analyses, we utilized both richness and inverse Simpson's diversity index metrics. We have deposited all scripts for analysis and figure generation in a publicly accessible GitHub repository (kvasir7/Graptemys_microbiome).

RESULTS

We analyzed a total of 30 individual cloacal swabs via 16S rDNA high-throughput sequencing: swabs from 6 turtles were analyzed from the James River site (5 females, 1 male), 11 turtles from the Goat Island site (10 females, 1 male), and 13 turtles from the Vermillion River site (8 females, 5 males). These 30 samples resulted in a total of 3,007,114 reads (1,503,557 paired-end constructs). Sample statistics indicated a mean paired-end construct count of 50,119/sample (Suppl. Fig. 1). We utilized a sex ratio of 7 males:23 females due to a sampling bias towards females. Sampled females had a mean carapace length of 185 ± 39 mm (mean ± 1 SD) and sampled males had a mean carapace length of 130 ± 7 mm, resulting in sampled turtles with an overall mean carapace length of 172 ± 41 mm (Table 1). A Kruskal–Wallis rank sum test determined no significant differences in carapace length among the three locations (H = 3.58, df = 2, P = 0.167; α = 0.05 hereafter).

Surface water temperatures were also taken and compared at each of the three locations. Our comparisons indicate a significant difference among all sites at time of sampling using a Kruskal–Wallis rank sum test (H = 18.2, df = 2, P = 0.000111). We followed with comparisons by site using Dunn's test for multiple comparisons using a Benjamini–Hochberg adjustment, which determined two of the three sites had significant pairwise differences (JimRiver–Goat Island Z = 1.16, df = 1, *P-adjusted* = 0.245; Vermillion River–Goat Island Z = 2.23, df = 1 *P-adjusted* = 0.0383; Vermillion River–JimRiver; Z = 4.11, df = 1, *P-adjusted*

TABLE 1. Summary of turtle metadata including location, water temperature (°C), secondary sex ratio, and straight-line carapace length (mm). Where appropriate, mean and standard deviation are given. [a] Water temperature was recorded at 4/11 Goat Island trapping sites.

Sampling location	Water temp. (°C; mean ± SD)	*n*	Sex ratio (M:F)	Female carapace length (mm; mean ± SD)	Male carapace length (mm; mean ± SD)	Combined carapace length (mm; mean ± SD)
James River	29.6 ± 0.2	6	1:5	176 ± 19	128	168 ± 26
Goat Island	26.0 ± 0.1[a]	11	1:10	161 ± 40	121	158 ± 39
Vermillion River	24.0 ± 1.1	13	5:8	216 ± 17	133 ± 6	186 ± 45
All sites	25.8 ± 2.5[a]	30	7:23	185 ± 39	130 ± 7	172 ± 41

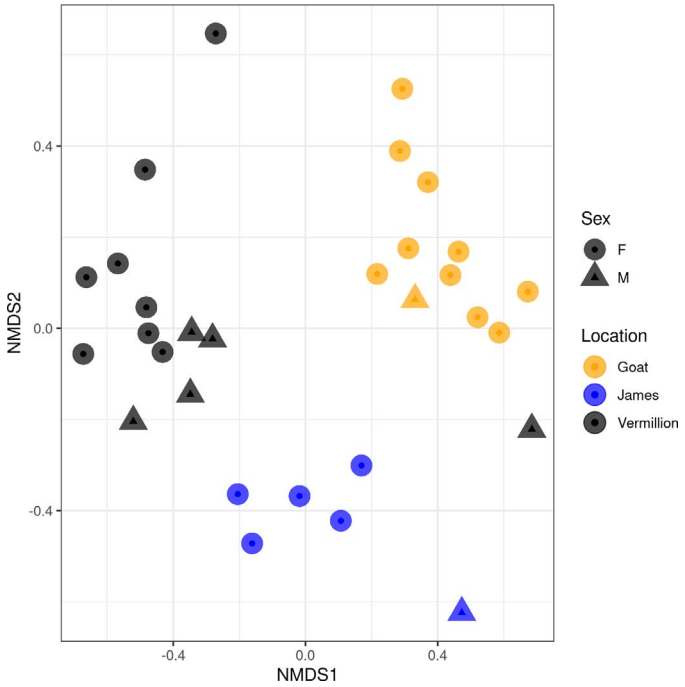

FIG. 2. Nonmetric multidimensional scaling calculated with the Bray-Curtis distance metric using a square root transformation and Wisconsin double-standardization. Location is represented by color, and sex is represented by shape. Stress of fit for the ordination is reported at 0.145. Axis titles represent the two dimensions to which the data have been ordinated.

= 0.000119). Corresponding National Weather Service–derived air temperature data in Sioux City, Iowa (ca. 48–80 straight-line km from sampling locations) indicate daytime high and nightly low ranges of 27–33°C and 14–22°C, respectively (NWS, 2020; Suppl. Table 1).

Microbial community α-diversity analyses among sampling locations showed similar species richness from one sample to another within sampling locations, with exceptions in the Vermillion River (Suppl. Fig. 2). Additionally, inverse Simpson's diversity metrics revealed qualitatively similar bacteria species distribution between samples from the same sampling location (Suppl. Fig. 2). Qualitative α-diversity analyses were followed by microbial community β-diversity analysis indicating a significant difference in composition among trapping locations (PERMANOVA: *pseudo-*$F_{2,27}$ = 3.29, r^2 = 0.196, P = 0.00100). Follow-up pairwise comparisons revealed significant differences between all sites (James River–Goat Island: $F(model)_{1,15}$ = 3.20, *P-adjusted* = 0.00600; Vermillion River–Goat Island: $F(model)_{1,22}$ = 3.61, *P-adjusted* = 0.00300; Vermillion River–James River: $F(model)_{1,17}$ = 2.98, *P-adjusted* = 0.00300). These differences also align with groupings seen in nonmetric multidimensional scaling visualization results (Fig. 2). A significant difference was also observed between sexes pooled from all locations (PERMANOVA: *pseudo-*$F_{1,28}$ = 1.80, r^2 = 0.0604, P = 0.0130), but this was likely the result of differences in group β-dispersion. Specifically, permutation tests were conducted to examine homogeneity of β-dispersion. Our results indicated significant differences between the sexes ($F_{1,28}$ = 16.338; P = 0.002), meaning the corresponding sex PERMANOVA result is likely reflecting differences in group dispersion and not centroid distribution. Location differences due to β-dispersion were also examined, and global significance was found ($F_{2,27}$ = 4.5; P = 0.025). However, a follow-up Tukey's HSD pairwise test showed only the Vermillion River–James River comparison having a significant difference in group dispersions, indicating significant differences in the other two pairwise comparisons as suggested by the respective location PERMANOVA result (James River–Goat Island: P = 0.471; Vermillion River–Goat Island: P = 0.140; Vermillion River–James River: P = 0.0217).

Community composition qualitative results indicate Proteobacteria comprised the plurality of bacteria phyla present across all locations, followed by Bacteroidetes in percent composition (Fig. 3). Even with these similarities, microbial community composition significantly varied by geographic location. Bacteria genera were also qualitatively examined (Fig. 4) with apparent differences in relative abundance by both site and individual that included a notable overrepresentation of Chryseobacterium at the James River site. Among similar genera represented, all sites had relatively high abundances of unclassified genera from the families Xanthomonadaceae, Burkholderiaceae, and Weeksellaceae.

DISCUSSION

The results of our study indicate that *G. pseudogeographica* cloacal bacteria community diversity varies across habitat types

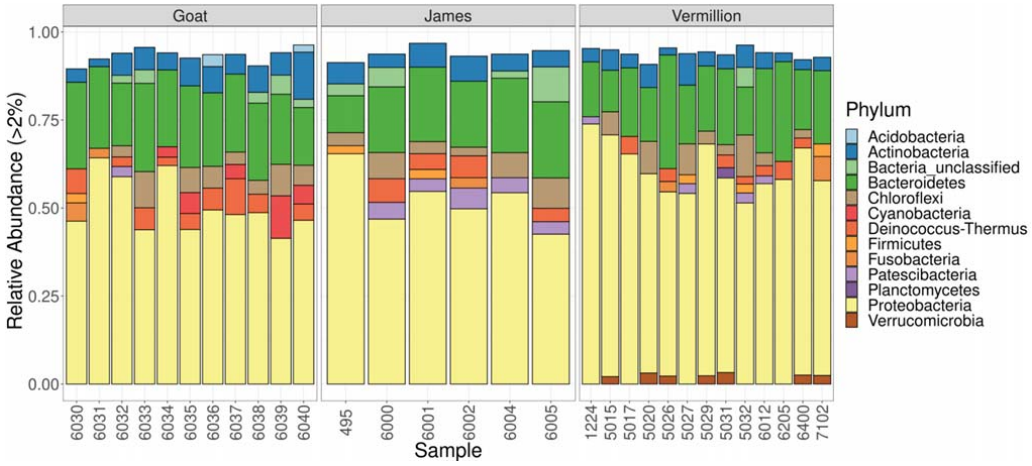

FIG. 3. Gut bacteria community relative abundances shown with each bar representing an individual sample in the corresponding location. Each color is representative of a corresponding phylum (or in the case of "Bacteria_unclassified," unidentified members of the bacteria). Bars remain unnormalized with remaining space composed of excluded low representation groups (≤2% of total abundance).

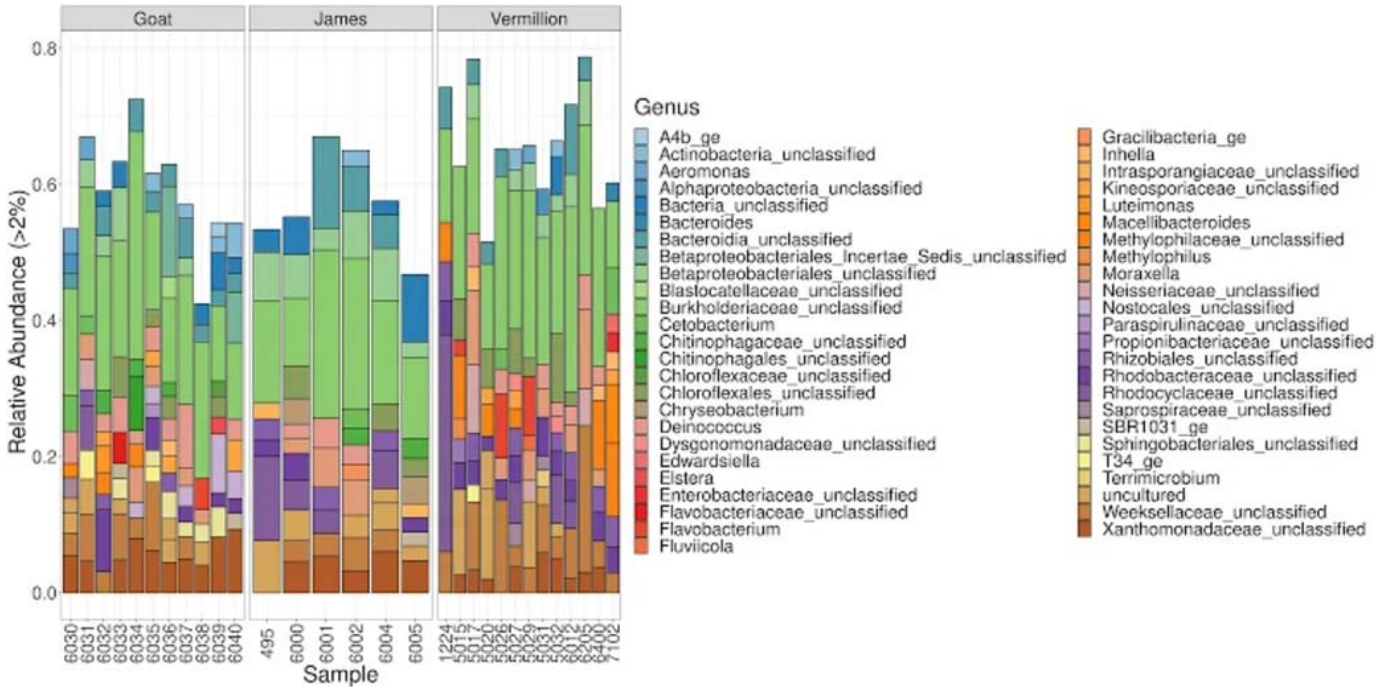

FIG. 4. Gut bacteria community relative abundances shown with each bar representing an individual sample in the corresponding location. Each color is representative of a corresponding genera. Where genera is not classified, the source family is given preceding "unclassified." Bars remain unnormalized with remaining space composed of excluded low-representation groups (≤2% of total abundance).

along the Missouri River. *Graptemys pseudogeographica* from Goat Island had different cloacal bacteria community β-diversity from individuals sampled at the confluences of the Vermillion River or James River. Our observed differences between sites are likely due to habitat and other environmental differences of an island within the main channel of the Missouri River versus proximity to a tributary confluence with the main channel. Both the James and Vermillion rivers have watersheds dominated by agricultural land use (corn and soybean row crops; Vogelmann et al., 2001), and as a result have reduced water quality parameters and increased levels of agricultural contaminants (USDA, 2009; SDDENR, 2020). At the river confluences, turtles remain exposed to contaminants before concentrations are likely to be diluted into the volumetrically larger mainstem of the Missouri River. Environmental differences among our study sites are therefore not unexpected and the results of our study may be attributable to such differences.

Among environmental variables, surface water temperature was the only metric that was measured in our study and that was shown to be significantly different among sampling sites, likely due in part to the aforementioned factors. While some differences may be due to differences in sampling time over a 28-d period, we do not think that the change was a major factor in the differences because climatic variables were relatively stable over the sampling period, and erosive processes associated with the planting and harvesting of crops is not a concern during midsummer. Seasonal differences within and among sites in surface water temperature are also possible and could be examined in future work. Other environmental factors associated with our observed differences, including concurrent sampling of the water microbiota and additional water quality data, were not systematically explored. The determination and correlative effects of these factors will require additional investigation.

The β-diversity of the bacterial microbiota in *G. pseudogeographica* at each of the trapping sites were significantly different based on a PERMANOVA comparing microbial composition among the three locations (omnibus). Follow-up pairwise comparisons indicated significant differences between all sites. Within-group β-dispersion was also examined and only found to be significant for the Vermillion River–James River comparison. Therefore, significant differences in bacterial microbiota were attributable to between-site differences at the James River–Goat Island and Vermillion River–Goat Island comparisons, and only in part at the Vermillion River–James River comparison (e.g., Warton et al., 2012). The extent to which intergroup variation exists among spatial gradients as opposed to strictly defined adjacent groups is not known. However, our work suggests that spatial differences exist and that spatial groups among riverine habitats cannot be assumed to have similar cloacal microbiota community diversity. Limited qualitative differences in α-diversity among sites also confirm such spatial differences. Specifically, there was qualitatively increasing heterogeneity among α-diversity in samples from the Vermillion River site as compared with other sites. The reason for the qualitative difference in α-diversity among sites is unclear as the Vermillion River watershed has similar land use to the James River. Follow-up work examining additional metrics of water quality and related hydrology between these sites may aid in better understanding the causes of the observed differences.

The β-diversity of the bacterial microbiota in *G. pseudogeographica* was found to be significantly different between the sexes, however this was likely the result of a significant difference in group dispersion. There was also a significant carapace-length difference observed by sex, indicating that size, as it relates to sex, has a negligible effect on microbial community diversity. We attempted to sample similar numbers of male and female *G. pseudogeographica* as determined by secondary sex characteristics (e.g., enlarged, elongate tails and

long forelimb claws in males), despite previous data suggesting a female-biased secondary sex ratio of *G. pseudogeographica* in the lower Missouri River (Lindeman, 2013). Specifically, the proportion of males (secondary sex ratios) within South Dakota populations has been reported as 0.21 and 0.27 (Lindeman, 2013). Despite our attempts to sample additional males, our sex ratios remained female skewed with only one male per site being represented at the Goat Island and James River sites, which precluded site-based statistical comparisons. We recommend that future studies on microbiome-related sex differences increase overall sample size to have the numbers required for including sex as a factor of analysis.

While bacterial microbiota differed among individuals, significant differences in bacterial microbiota metacommunity structure, when compared with a neighboring population less than 48 km away, may require further explanation of the impact of local habitat on microbial fauna within aquatic species, particularly in a riverine habitat. Movement ranges for *G. pseudogeographica* have not been explicitly measured, but data exists recording movement distances ranging from 1.2 to 8 km (Vogt, 1980). Given the documented turtle movement distances, we suggest that turtles sampled from each of the three populations had higher intrapopulation genetic relatedness than interpopulation. We therefore treated the various trapping sites as different geographic groups that likely constitute different genetic subpopulations, although a more detailed study of *G. pseudogeographica* spatial relatedness is clearly needed. However, innate genetic differences among host groups are not thought to be deterministic towards the associated microbial communities. Rather, and as broadly proposed elsewhere, we suggest that host–environment interactions at various evolutionary and ecological levels constitute the observed diversity differences (Gould, 2002). While similar work has found significant population differences in Green Sea Turtle rectal microbiota diversity based on feeding modes related to habitat (i.e., herbivorous vs. omnivorous; Campos et al., 2018; Bloodgood et al., 2020), our study is the first to examine the impact of geographic location on host-associated microbiota on a small scale and to find significant differences.

The microbial taxonomic results of our study are reflective of taxa that would be expected in an aquatic reptile. The phylum Proteobacteria was the most abundant phyla of bacteria across all samples, a pattern that is in agreement with studies done on other aquatic reptiles including the Green Sea Turtle (Price et al., 2017), the Loggerhead Sea Turtle (Abdelrhman et al., 2016), and the American Alligator (*Alligator mississippiensis*; Keenan et al., 2013). The phylum Bacteroidetes was also identified in all samples collected, again coinciding with results found in the Green Sea Turtle and Loggerhead Sea Turtle. Many other phyla were found in community composition analysis in very low abundance, but were found across all trapping sites, including Actinobacteria, Chloroflexi, and Deinococcus-Thermus. Our observed phyla, as well as those found in higher abundances including Proteobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia, were expected. The observed phyla are commonly found in microbial community samples from natural habitats (Costello and Schmidt, 2006; Fierer et al., 2007; Bergmann et al., 2011; Santhanam et al., 2017), as compared with the different phyla often found in captive animals (Kohl et al., 2014; Madison et al., 2018). The genera that were identified in our samples, though diverse, are also reflective of those found in previous work. Nonetheless, there were some qualitative differences among sites including an overrepresentation of Chryseobacterium at the James River site, as well as varying abundances of Flavobacteriaceae. Taxa of the Chryseobacterium and the Flavobacteriaceae are of interest because of their known pathogenesis of aquatic organisms and broad antibacterial resistance (Kumru et al., 2020). We do not provide statistical comparisons of the differing abundances due to unresolved issues of false discovery (Hawinkel et al., 2019). However, we suggest that future examination of specific bacterial taxa at the genus and family level may be useful as a proxy for multistudy comparisons of key marker taxa indicative of host and ecosystem health.

While reflective of what is expected, the use of cloacal swabs as opposed to gut or fecal sampling may yield different results in future work and should also be considered when comparing studies. Cloacal swabs were used in our study largely due to their less invasive character (vs. sacrificing individuals for gut sampling), and their comparative potential to previous work as highlighted. While potentially yielding different results in community composition from other host body regions, we argue that various host-associated microenvironments can produce different respective microbial communities. The different communities of bacterial microbiota may play a role in host health and should therefore be considered. However, the cloaca likely has a high degree of microbial exchange with the environment when compared with the gut, and therefore better represents potential environmental effects on host health via microbial communities. Cloacal swabbing is an informative methodology; however, any swabbing and related taxonomic comparisons will be context and goal dependent.

In addition to host body region and sampling techniques, various environmental factors also have a likely role in determining the bacterial microbiota found in our study. One factor of interest that may be determinant under natural conditions is the movement of soil dwelling bacteria to riverine habitats. Proteobacteria are one candidate for such movement and were also the most abundant taxa in the microbiota of previously studied species (Keenan et al., 2013; Abdelrhman et al., 2016; Price et al., 2017). Proteobacteria also had a qualitatively higher representation at the Vermillion River site as compared with other sites. Verrucomicrobia were also detected among locations and likewise overrepresented in Vermillion River samples. The presence of Proteobacteria across all samples, and Verrucomicrobia in most, may therefore be partly attributed to soil erosion and subsequent interhabitat transfer, thereby increasing the abundance of Proteobacteria and Verrucomicrobia already found in aquatic environments. The increased levels of Proteobacteria and Verrucomicrobia among samples from the Vermillion River site may therefore indicate higher sedimentation in the river, corresponding with higher host abundance. How such events could affect host-associated microbiota and host health should be a focus of future work.

We have shown that geographic location has a significant relationship to the cloacal microbiota diversity of wild-caught *G. pseudogeographica*. The exact reasons for location affecting microbiota diversity remain unclear but showing a significant relationship between the two acts as a first step for further study into this correlation. Given *G. pseudogeographica* is a state-threatened species in South Dakota, our data serve as an information baseline to monitor future changes in bacterial microbiota, particularly in response to river management and changes in habitat quality. Pathogens are also known to be involved in host–microbiota dynamics. While pathogens may

be involved in determining the observed results, we previously reported no ranavirus-positive individuals in our study populations, including in these 30 specimens (Butterfield et al., 2019). We also suggest that further study on the impact of water quality factors on microbial community composition of wild-caught turtles should take into consideration the differences noted in community structure between captive and wild turtles. As we learn more about the impact of the microbiota on the health of organisms, populations, and the ecosystems they interact with, research concerning both the effects of microbiota and how it is influenced are of critical importance for the conservation of threatened turtle species such as *G. pseudogeographica*.

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Data Accessibility.—Sequencing files used in this study have been deposited in the Sequence Read Archive (SRA) with accession numbers SRX8265363–SRX8265382. All R scripts used in data analysis and figure production can be found on GitHub (kvasir7/Graptemys_microbiome). Field data is available as a supplemental file.

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Supplementary Data

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