

Kinship, inbreeding and fine-scale spatial structure influence gut microbiota in a hindgut-fermenting tortoise

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Abstract

Herbivorous vertebrates rely on complex communities of mutualistic gut bacteria to facilitate the digestion of celluloses and hemicelluloses. Gut microbes are often convergent based on diet and gut morphology across a phylogenetically diverse group of mammals. However, little is known about microbial communities of herbivorous hindgut-fermenting reptiles. Here, we investigate how factors at the individual level might constrain the composition of gut microbes in an obligate herbivorous reptile. Using multiplexed 16S rRNA gene sequencing, we characterized the faecal microbial community of a population of gopher tortoises (*Gopherus polyphemus*) and examined how age, genetic diversity, spatial structure and kinship influence differences among individuals. We recovered phylotypes associated with known cellulolytic function, including candidate phylum Termite Group 3, suggesting their importance for gopher tortoise digestion. Although host genetic structure did not explain variation in microbial composition and community structure, we found that fine-scale spatial structure, inbreeding, degree of relatedness and possibly ontogeny shaped patterns of diversity in faecal microbiomes of gopher tortoises. Our findings corroborate widespread convergence of faecal-associated microbes based on gut morphology and diet and demonstrate the role of spatial and demographic structure in driving differentiation of gut microbiota in natural populations.

Keywords: 16S rRNA sequencing, faecal microbiota, gopher tortoise, *Gopherus polyphemus*, microbial diversity, microsatellites

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Introduction

Gut microbial communities are often characteristic of specific dietary modes (Ley *et al.* 2008; Muegge *et al.* 2011; Delsuc *et al.* 2014). For example, obligate herbivory in mammals is associated with increased microbial diversity relative to other dietary modes and the dominance of cellulolytic lineages (Ley *et al.* 2008), highlighting the inability of vertebrates to digest cellulose. The mutualism between cellulolytic bacteria and vertebrate herbivores has proven immensely successful, as herbivores constitute over 80% of all mammals (Stevens & Hume 2004). However, obligate herbivory is less widespread in nonavian reptiles, although it has

evolved independently in tortoises (King 1996), skinks (Herrel 2007) and several lineages of iguanids and teiids (Troyer 1983; Espinoza *et al.* 2004; Vitt 2004). Despite the rarity of herbivory among reptiles, their digestive efficiency is comparable to hindgut-fermenting mammalian herbivores (Bjorndal 1987; Hatt *et al.* 2005). Galápagos tortoises and several iguanids share a similar digestive mode and gut morphology with mammalian hindgut fermenters (Bjorndal 1997) and have a similar faecal community composition at high taxonomic levels (Ley *et al.* 2008; Hong *et al.* 2011). Therefore, the convergence in gut microbial communities among herbivorous mammals may also extend to herbivorous reptiles; however, we know very little about species-specific factors that might constrain the composition of gut microbes in hindgut-fermenting reptiles.

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Although species identity and phylogenetic history are important sources of variation in patterns of gut microbiota diversity, there are also notable intraspecific factors such as individual diet (Turnbaugh *et al.* 2008, 2009; Filippo *et al.* 2010; Bolnick *et al.* 2014c), host genotype (Benson *et al.* 2010; Kovacs *et al.* 2011; Bolnick *et al.* 2014a), geographic location (Fallani *et al.* 2010; Yatsunenکو *et al.* 2012; Linnenbrink *et al.* 2013), sex (Fierer *et al.* 2008; Markle *et al.* 2013) and age (Hopkins *et al.* 2002; Mariat *et al.* 2009). For example, Galápagos land and marine iguanas show geographic structure in gut microbiota across islands (Lankau *et al.* 2012). Similarly, humans exhibit geographic differences in gut microbiota (Fallani *et al.* 2010; Yatsunenکو *et al.* 2012), although these effects are confounded by overlapping differences in culture, sanitation and genetics (Lozupone *et al.* 2012). In addition, humans show ontogenetic changes in gut microbial communities, typically with juvenile microbial communities being less diverse and less stable than adult microbial communities (Hopkins *et al.* 2002; Jiménez *et al.* 2008; Mariat *et al.* 2009; Koenig *et al.* 2011; Funkhouser & Bordenstein 2013). In humans, a generalist omnivore, a high degree of individual variation exists in gut microbial community composition and structure. While changes in health and diet can influence an individual's gut community, such variation is small relative to natural interpersonal variation (Lozupone *et al.* 2012). Whether such variation is common among species with more restricted lifestyles and diets remains an open question.

Kinship plays an important role in shaping gut microbial communities in several species either through direct maternal transmission to offspring *in utero* (vertical transmission), or through parental care and association with related conspecifics (horizontal transmission) (Lombardo 2008; Funkhouser & Bordenstein 2013). Distinct microbial communities transmitted from mothers to offspring can persist to adulthood, at least in some vertebrate species (Nelson *et al.* 2013). In humans, initial horizontal transmission of gut microbes from mothers to infants occurs during childbirth, as well as through contact during maternal care (Funkhouser & Bordenstein 2013). Similarly, in turtles, several bacterial species can be maternally transmitted either through the cloaca or potentially via egg fluids or membranes prior to complete shell formation (Al-Bahry *et al.* 2009; de Moraes *et al.* 2010). However, documented maternal transmissions of bacteria in turtles have focused on a few potentially pathogenic species rather than overall gut communities. Horizontal maintenance of microbial communities can also potentially contribute to the evolution of kin association and, in some cases, sociality (Troyer 1984a; Lombardo 2008). Bacteria can produce chemical cues, both attractants and deterrents, which may shape

social interactions and facilitate kin recognition and microbial transmission in host species (Archie & Theis 2011). Green iguana (*Iguana iguana*) hatchlings exhibit horizontal kin-directed coprophagy, which transfers cellulolytic microbes from related adults to neonates (Troyer 1982, 1984b). Although transmission via coprophagy has been documented in green iguanas, it has not been clearly demonstrated in tortoises.

Here, we investigate factors that influence faecal microbial diversity in a Florida population of threatened gopher tortoises, *Gopherus polyphemus*. Gopher tortoises are efficient hindgut fermenters (Bjorndal 1987) with social interaction (Guyer *et al.* 2014), but lack parental care. Although several species of tortoises exhibit coprophagy even in the presence of abundant food (Burchfield *et al.* 1980; Lance & Morafka 2001; Joshua *et al.* 2010), it is unknown whether this behaviour is the primary route of colonization for critical gut symbionts. The gut microbiota of gopher tortoises has been examined in northern populations (Louisiana, Alabama, Mississippi and Georgia), and the dominant phyla are similar to other herbivorous reptiles (Gaillard 2014). Here, we focus instead on a southern Florida population of tortoises and on understanding the intraspecific factors influencing microbial community composition and structure. Specifically, we aim to (i) characterize the core faecal-associated microbiota of *G. polyphemus*, and determine whether their faecal microbiota show (ii) variation across fine geographic scales due to habitat variation, (iii) differences associated with host inbreeding, (iv) evidence of transmission based on kinship and (v) ontogenetic shifts in community composition and structure. Our results contribute to understanding evolutionary trends in herbivory and associated gut microbiota, as well as the underlying dynamics of gut community composition and structure in herbivorous reptiles.

Methods

Study population and sample collection

Our study population of *G. polyphemus* is located at Archbold Biological Station on the Lake Wales Ridge in south-central Florida. The tortoises at our study site are part of a naturally occurring population located within the historic range of the species. Although 44 tortoises were translocated to the station between 1968 and 1981, recapture rates of translocated individuals were very low and none were observed after 14 years despite continued monitoring, suggesting all had emigrated or died (Layne 1989). The study site is a research reserve divided into management units subject to different fire regimes. We nondestructively sampled tortoises from four adjacent management units (Fig. 1). Although all sampled



Fig. 1 Map of sampled units at Archbold Biological Station. Location of Archbold Biological Station is marked by a star in the inset map of Florida. Old State Road 8, which divides unit Main Drive (MD) from Hill Garden (HG), Hill-West (HW) and Hill-East (HE), is labelled.

tortoises come from a single population, we treated each management unit as a separate entity due to their differing fire regimes, soil types and land-use histories. The Main Drive (MD) unit is separated from the remaining management units by a highway (Fig. 1), which is a permeable barrier to tortoise movement (B. B. Rothermel, personal observation). All tortoises from MD were sampled from grassy road shoulders surrounded by mixed oak–hickory scrub and scrubby flatwood vegetation. The Hill Garden (HG) unit was cleared in the 1930s and planted with a variety of exotic species; it is currently maintained as old-field habitat dominated by bahiagrass (*Paspalum notatum*). The surrounding Hill-East (HE) and Hill-West (HW) are southern ridge sandhill habitats treated with different fire management regimes, resulting in different composition and densities of vegetation (Table S1, Supporting information). The tortoise population density within HG (~6.1 tortoises/ha) far exceeds the surrounding management units (e.g. HE, ~1.4 tortoises/ha; B. B. Rothermel, unpublished data), which are more typical of inland scrub habitats in peninsular Florida (1.3–3.0 tortoises/ha; Castellón *et al.* 2012).

We nondestructively sampled blood ($N = 45$) and faeces ($N = 46$) from captured tortoises. As a reference data set for estimating allele frequencies, we collected only blood from 54 additional tortoises. We captured tortoises by hand or using Havahart live animal traps (Woodstream Corporation, Lititz, PA, USA) placed at burrow entrances. Throughout 2012–2014, we collected 0.5–1.0 mL of blood via the subcarapacial vein (Hernandez-Divers *et al.* 2002) and stored it in lysis buffer (100 mM Tris base, 100 mM EDTA, 150 mM NaCl, 1% SDS). We collected faecal samples (1–2 g) during August 2013 from the interior of stools immediately

after the tortoises defecated onto sterile trays. Faecal samples were frozen at $-20\text{ }^{\circ}\text{C}$ on site and subsequently stored at $-80\text{ }^{\circ}\text{C}$. As part of long-term population monitoring, tortoises in our study area are individually marked. For each individual, we determined sex, measured carapace length at time of sample collection and compiled all available capture locations between 2009 and 2013. Sex was determined using secondary sexual characteristics (McRae *et al.* 1981), observation of breeding behaviours, or penis extrusion. Because we do not have records of exact age for most tortoises, we used body size as a proxy for age and sorted individuals into either juveniles (i.e. prereproductive individuals) or adults. Tortoises were considered mature if their carapace length was $>230\text{ mm}$ for males and $>255\text{ mm}$ for females (Landers *et al.* 1982).

Pairwise geographic distance

We determined pairwise proximity between tortoises using home burrows (for females) or centroids of home ranges (for males). Whenever possible, we determined female home burrows based on camera monitoring between June and November 2013 (S. H. Dean, unpublished data). We could not assign male tortoises to burrows due to their extensive use of multiple burrows and their frequent visitation of females (Eubanks *et al.* 2003). Therefore, we used aggregated capture data between 2009 and 2013 to infer minimum convex polygon home ranges and centroids in ARCGIS 10 (ESRI, Redlands, CA, USA). We determined pairwise geographic distance between tortoise home burrows or centroids of home ranges in GENALEX 6.5 (Peakall & Smouse 2012).

Microsatellite analysis

We extracted whole genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands) and polymerase chain reaction (PCR)-amplified each sample at 24 previously published microsatellite markers (Schwartz *et al.* 2003; Tuberville *et al.* 2011; Kreiser *et al.* 2013) (Table S2, Supporting information). We conducted PCRs using a three-primer system (Waldbieser *et al.* 2003) consisting of published primer pairs and a third fluorescently tagged universal primer. We attached a 5'-CGAGTTTTCCCAGTCACGAC-3' universal tag to the 5' end of either microsatellite primer and 5'-GTTT-3' tag to the 5' end of the untagged primer to reduce stutter. Fluorescently tagged universal primers were designed by attaching VIC, NED, PET or 6-FAM to the 5' end of the universal tag sequence (5'-CGAGTTTTCCCAGTCACGAC-3').

We performed all PCRs in 10 μ L reactions including 1 μ L template DNA (1–10 ng), 1 \times PCR buffer, 1.5 mM MgCl₂, 0.1 μ M dNTPS, 0.2 μ M each of untagged and universal fluorescent primers, 0.4 μ M tagged primer and 0.25 units of *Taq* polymerase (Roche, Basel, Switzerland). PCRs consisted of an initial denaturing temperature of 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 1 min, locus-specific annealing temperature for 1 min (see Table S2, Supporting information), extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. We then pooled our PCR products in sets of four with one of each fluorescent primer. We mixed 1 μ L of pooled PCR product with 18.85 μ L Hi-Di™ formamide and 0.15 μ L GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems, Foster City, CA, USA). We genotyped samples using an ABI 3730XL DNA ANALYZER (Applied Biosystems). We scored alleles using GENEMARKER v2.4.0 (SoftGenetics, State College, PA, USA) and visually confirmed all calls.

For each pair of tortoises, we calculated the Queller–Goodnight (QG; Queller & Goodnight 1989) estimator of relatedness (r) using COANCESTRY v1.0.1.1 (Wang 2011). Because we lack a known outbred reference population, we estimated allele frequencies from our reference data set of 54 nonfocal individuals. Focal individuals were excluded from allele frequency estimation to reduce sampling error (Wang 2014). Because relatedness estimators cannot be interpreted as probabilities of identity by descent when allele frequencies are estimated directly from the study population, we chose the QG estimator because it can be interpreted as a correlation coefficient of homologous genes (Wang 2014). Additionally, we calculated individual inbreeding coefficients (F) in COANCESTRY using the Ritland moment estimator (Ritland 1996). As a second metric of kinship, we implemented a maximum-likelihood method in COLONY

v2.0.5.8 (Jones & Wang 2009) to assign dyads to one of three categories: (i) full-sibling or equivalent, (ii) half-sibling or equivalent, or (iii) unrelated. These designations do not mean that a given dyad is a full-sibling or half-sibling pair; rather, the categories include all dyads with any relationship equivalent to sibling-level relatedness ($r = 0.5$) or half-sibling relatedness ($r = 0.25$).

For each management unit and the overall population, we calculated observed and expected heterozygosity in ARLEQUIN v3.5 (Excoffier & Lischer 2010), as well as rarefied allelic and private allelic richness in HP-RARE (Kalinowski 2005). To examine potential underlying genetic structure in our host population, we determined the number of genetic clusters among our samples using STRUCTURE v2.3.4 (Pritchard *et al.* 2000) including both the focal and reference microsatellite data sets, but excluding individuals we could not reliably assign to a management unit. Our reference data set includes management units not sampled in the microbiome study and thus increases the spatial scale of sampling, placing genetic differences within our focal samples in a broader context. We implemented four replicates for each number of putative clusters ($K = 2$ –8) using a burn-in period of 100 000 and 1 000 000 Markov chain Monte Carlo simulations. We chose the optimal number of clusters using the Evanno *et al.* (2005) criteria determined by STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt 2012). We visualized STRUCTURE results using CLUMPAK (Kopelman *et al.* 2015). Additionally, we calculated pairwise F_{ST} among management units.

Multiplexed 16S rRNA gene sequencing

We extracted whole community microbial genomic DNA using PowerSoil® DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) following the Earth Microbiome Project protocol (<http://www.earthmicrobiome.org>; Caporaso *et al.* 2012). All extractions were quantified using a QUBIT® 2.0 (Invitrogen, Carlsbad, CA, USA) and then diluted to between 1 and 5 ng/ μ L of DNA. We included two double-distilled water (ddH₂O) samples as controls to detect potential contamination from reagents or methodology.

For each sample, we conducted triplicate PCR amplifications using the universal 515F and Golay barcoded 806R 16S ribosomal RNA V4 region primers (Caporaso *et al.* 2012). PCR conditions followed Caporaso *et al.* (2011). Briefly, we performed reactions using 0.2 μ M of each primer, 10 μ L of 5 Primer Hot Master Mix (5 Prime, Gaithersburg, MD, USA) and 1 μ L of diluted template DNA. PCRs were carried out at an initial denaturing temperature of 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at

50 °C for 1 min, extension at 72 °C for 1.5 min and a final extension at 72 °C for 10 min. Triplicated PCRs were pooled, visualized on a 2% agarose gel and quantified using a QuantiFluor® dsDNA system (Promega, Madison, WI, USA). We then combined 100 ng of each sample and cleaned the pooled product using the ChargeSwitch® PCR Clean-Up Kit (Invitrogen). Gut microbial communities were sequenced using 2 × 250 paired-end reads on a MiSeq benchtop sequencer (Illumina, San Diego, CA, USA).

We performed downstream data analysis using QIIME v1.8.0 (Caporaso *et al.* 2010). Paired-end reads were joined (forward and reverse reads were compiled into a single sequence), and sequences were filtered using default parameters in QIIME. We discarded putatively chimeric sequences identified using UCHIME (Edgar *et al.* 2011). We used UCLUST (Edgar 2010) to cluster sequences into operational taxonomic units (OTUs) with 97% minimum identity. We performed microbial taxonomic assignment using the RDP Classifier (Wang *et al.* 2007), a naïve Bayesian algorithm, trained to the GreenGenes database (DeSantis *et al.* 2006; McDonald *et al.* 2012). We filtered out singleton OTUs, as they likely represent PCR errors, and determined the core microbial community by filtering OTUs that did not appear in at least 90% of samples.

Microbial community analyses

We conducted community analyses in QIIME, which utilizes R v3.0.3 (R Development Core Team 2014) in its implementation. All samples were rarified to 112 500 reads to control for potential artefacts of sequencing depth in downstream analyses. For each sample, we calculated phylotype richness, phylogenetic diversity and the Simpson index. The Simpson index allowed us to examine the role of relative abundances, while phylogenetic diversity examined phylogenetic breadth. We compared alpha diversity indices (diversity within a community) across categorical variables (age class, sex, management unit, genetic cluster) and tested for correlations with continuous predictors (carapace length, inbreeding) using permutation tests. *P*-values for all permutation tests were determined using 999 iterations. For analyses considering sex, we excluded nonadult tortoises due to our inability to identify their sex. When testing the influence of nuclear genetic clusters on gut microbial communities, we only included individuals whose ancestries were assigned at ≥ 0.75 to a single genetic cluster to control for admixture. We examined which microbial OTUs (putative species) were differentially represented using either a nonparametric Kruskal–Wallis test or permutation tests for each variable. We also tested for correlations between genera and families with relative abundances above 1% and continuous variables, as well as family and generic differ-

ences across categorical variables, to account for the possibility that changes were driven by cumulative shifts across phylogenetically associated OTUs. Due to the large number of comparisons, results were corrected for multiple comparisons using the false discovery rate method.

To investigate patterns of beta diversity (differentiation among communities) among tortoises, we calculated pairwise faecal microbiome similarity using the unweighted and weighted UniFrac distance metric (Lozupone & Knight 2005). We applied both methods because they are differentially affected by rare (unweighted) or abundant (weighted) OTUs. To ensure that phylogeny was not masking potential effects, we also implemented the Bray–Curtis dissimilarity matrix (Bray & Curtis 1957). To test the hypothesis that kinship and geographic distance are correlated with gut community similarity, we employed partial mantel tests between each distance metric and either pairwise relatedness or geographic distance, controlling for the untested variable. As an additional test of kinship, we compared microbial community distances across COLONY-based kinship categories ('full-sibling', 'half-sibling' and unrelated) using permutation tests for differences between means. Specifically, we compared the observed differences among means to differences among null distributions of microbial community distances generated by repeated random sampling of the equivalent number of dyads for each kin category from all possible dyads (999 iterations).

To parse the influence of demographics, space and genetic diversity on microbial community composition, we performed principal coordinate analyses (PCoA) based on each distance metric. We then performed a permutational multivariate analysis of variance using distance matrices implemented in the R vegan package v2.0-10 (Oksanen *et al.* 2013). We used the function *adonis* to examine the effect of categorical (sex, age class, management unit, genetic cluster) and continuous (carapace length, inbreeding) variables on both unweighted and weighted UniFrac metrics, as well as the Bray–Curtis dissimilarity matrix. Significance was determined via a permutation test. To check whether our results were influenced by differential dispersion (unequal variances), we employed function *permdisp* for all categorical variables. Lastly, we further tested the influence of continuous variables (carapace length, inbreeding) though permutation tests comparing the correlation of each variable with the first two principal coordinates for a given distance metric.

Results

Across all samples, we recovered 8 352 124 reads after quality filtering. Individual samples ranged from

126 352 to 215 500 reads. These reads were assigned to 14 104 unique phylotypes (OTUs) representing 434 genera. Additional filtering for the core microbiome reduced the number of OTUs to 1470, representing 296 genera. Both ddH₂O controls had low numbers of reads ($N = 4713$ and $11\,220$) that were dominated by Proteobacteria (90–93% of reads). All potential contaminant OTUs were present in tortoise samples at <0.001% or were not detected.

Core microbiome

We recovered 1470 core OTUs, defined as those present in at least 90% of samples. The dominant phyla in the core gopher tortoise faecal microbiome were Firmicutes (36.0%) and Bacteroidetes (36.5%), which consistently dominated all samples (Fig. 2). Minor phyla that exceeded 1% abundance were Euryarchaeota (Archaea), candidate phylum Termite Group 3, Spirochaetes, Tenericutes, and Verrucomicrobia. Among Firmicutes, more than 97% of the recovered OTUs were members of the Clostridia compared to 0.8% Erysipelotrichi and 0.05% Bacilli, and the remainder belonging to unnamed/unknown classes (Fig. 2). Most Firmicutes were associated with the families Ruminococcaceae, Lachnospiraceae and unassigned Clostridiales. Less common Firmicutes families included Synthrophomonadaceae, Clostridiaceae and Christensenellaceae. The majority of Bacteroidetes in adults represent a single OTU within

the Bacteroidales (79.7%), but for which taxonomic information below the order level is not known. This single Bacteroidales member (GreenGenes reference #1878) was also the most abundant OTU overall, constituting approximately one-quarter of all reads. No other single OTU exceeds even 5% overall relative abundance. Archaea belonged to the genera *Methanobrevibacter*, *Methanosphaera* and *Methanospirillum*. Additionally, we recovered other unclassified members of the Methanobacteriaceae and Methanocorpusculaceae, as well as a single group of Thermoplasmata, *vadinCA11* (Fig. 2).

Host population genetics

Overall population allelic richness was 4.76 with management units ranging from 4.46 to 4.92. Observed heterozygosity ranged from 0.542 to 0.606, and expected heterozygosity ranged from 0.607 to 0.634 across management units (Table 1). F_{ST} values were consistently low, ranging from 0 to 0.003, and none of the management units were significantly different from each other (all $P > 0.05$). Bayesian assignment recovered three genetic clusters within our population; however, individuals assigned to these genetic clusters were broadly overlapping and not spatially segregated by management unit (Fig. 3). We did not recover evidence of genetic outliers that would indicate the potential presence of descendants of translocated individuals. All

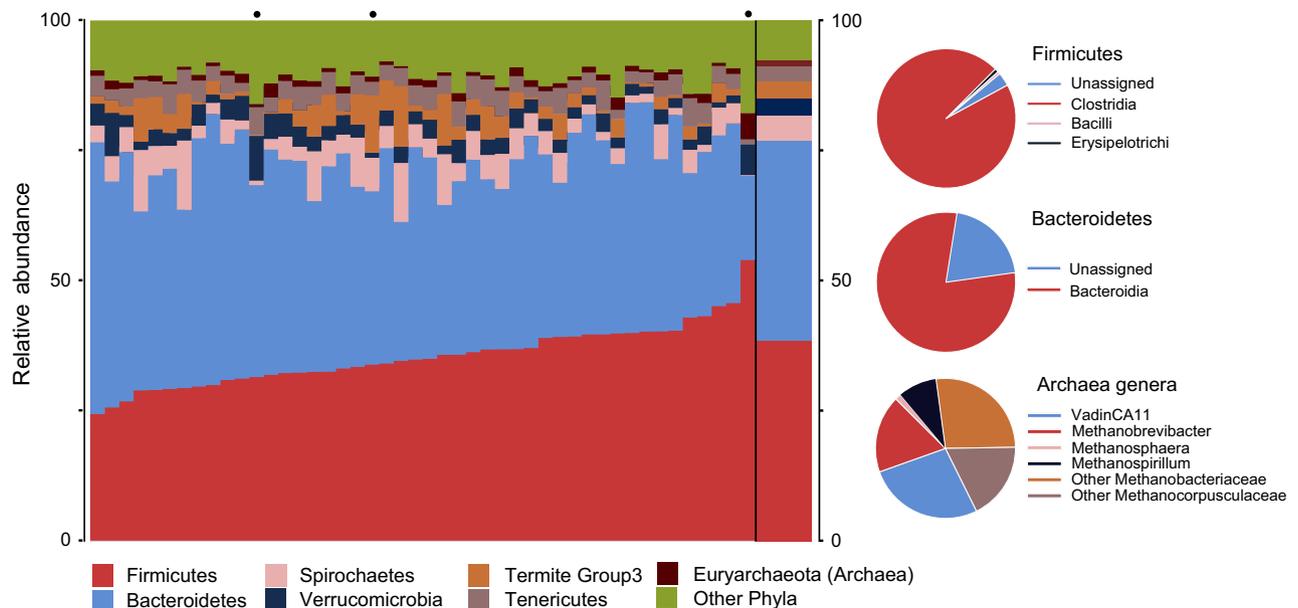


Fig. 2 Bar charts representing core gut bacteria community composition (phyla level) of *G. polyphemus* based on all 46 individuals. Juvenile individuals are denoted by a black dot above the bar. The average across all individuals is displayed to the right of the black line. Pie charts depict relative abundances of Firmicutes and Bacteroidetes classes, and Archaean genera. Bacterial OTUs with relative abundances of <3% are grouped into 'Other' categories to facilitate ease of viewing.

Table 1 Sample size (N), number of additional genetic reference samples, observed heterozygosity (H_O), expected heterozygosity (H_E), rarefied allelic richness (A) and private allelic richness (A_{pr}) for each management unit and the overall population

	N	# reference samples	H_O	H_E	A	A_{pr}
Hill Garden (HG)	30	7	0.589 ± 0.19	0.607 ± 0.24	4.62	0.13
Hill-East (HE)	5	20	0.560 ± 0.22	0.624 ± 0.21	4.92	0.38
Hill-West (HW)	4	8	0.542 ± 0.21	0.608 ± 0.23	4.75	0.29
Main Drive (MD)	7	2	0.606 ± 0.25	0.634 ± 0.20	4.46	0.43
Overall	46	37	0.572 ± 0.19	0.615 ± 0.21	4.76	–

Diversity indices were calculated using both reference and focal individuals collected from management units used in this study.

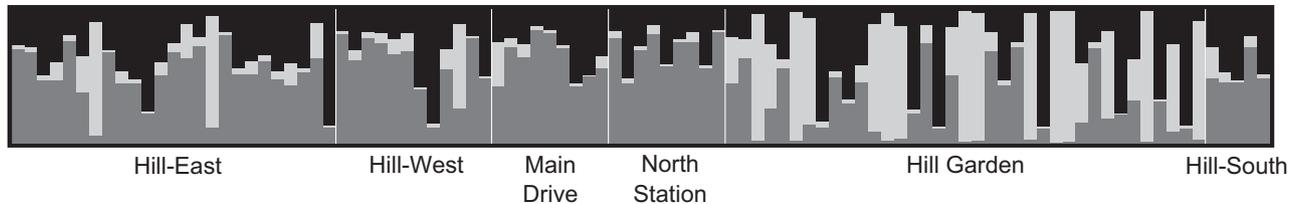


Fig. 3 STRUCTURE plot showing number of genetic clusters ($K = 3$) across focal and reference samples. Each column represents an individual with each shade corresponding to the proportion ancestry assigned to each genetic cluster. Samples are grouped by management units, separated by white lines. North Station and Hill-South consisted only of reference samples and were not sampled for the microbiota study.

clusters recovered by STRUCTURE include individuals known to be native to the site.

Patterns of alpha diversity

Between host age classes, we detected a significant difference in microbial phylogenetic diversity (permutation test for group differences: $P = 0.001$) and observed richness ($P = 0.001$). In all cases, juvenile communities were less diverse than adults. We found significant positive correlations of richness (permutation test for correlation: $R^2 = 0.238$, $P = 0.001$) and phylogenetic diversity with carapace length ($R^2 = 0.334$, $P < 0.001$). However, when juvenile tortoises ($N = 3$) were excluded, we did not find correlations with carapace length ($P > 0.05$). Simpson's index was negatively correlated with the inbreeding coefficient ($R^2 = 0.105$, $P = 0.017$) (Fig. 4). We found no differences associated with sex, genetic cluster or management unit ($P > 0.05$).

Patterns of beta diversity

Although we did not recover any OTUs whose relative abundances differed across tested variables after false discovery rate correction ($P > 0.05$), we recovered several bacterial families and genera whose relative abundances were significantly correlated with carapace length, and a single family (R4-41B) marginally correlated with inbreeding. Additionally, we recovered

genera, but not families, whose relative abundances differed across management unit and age class (Tables 2 and S3, Supporting information).

We found no correlation between microbial beta diversity and host relatedness or geographic proximity (partial mantel test: $P > 0.05$), no effect of sex or genetic cluster on beta diversity (*adonis*: $P > 0.05$), and no difference between 'half-sibling' and unrelated pairs for all distance metrics (permutation test: $P > 0.5$). However, unweighted UniFrac distances and Bray–Curtis distances were smaller, and weighted UniFrac distances marginally smaller, in 'full-sibling' pairs compared to both 'half-sibling' (unweighted, $P = 0.004$; Bray–Curtis, $P = 0.043$; weighted, $P = 0.097$) and unrelated pairs (unweighted, $P = 0.002$; Bray–Curtis, $P = 0.020$; weighted, $P = 0.057$) (Fig. 4). We observed a marginal effect of inbreeding on Bray–Curtis dissimilarity (*adonis*: $R^2 = 0.032$, $P = 0.093$) and weighted UniFrac distances ($R^2 = 0.089$, $P = 0.093$). We also found a significant effect of carapace length (unweighted, $R^2 = 0.044$, $P < 0.001$; weighted, $R^2 = 0.113$, $P = 0.002$; Bray–Curtis, $R^2 = 0.104$, $P = 0.002$) and age class (unweighted, $R^2 = 0.041$, $P < 0.001$; weighted, $R^2 = 0.131$, $P = 0.002$; Bray–Curtis, $R^2 = 0.098$, $P < 0.001$) on both UniFrac metrics. Finally, we detected a significant effect of management unit on unweighted UniFrac distances ($R^2 = 0.079$, $P = 0.002$) and Bray–Curtis dissimilarity ($R^2 = 0.096$, $P = 0.024$) (Fig. 5). Although we found significantly different unweighted UniFrac variances

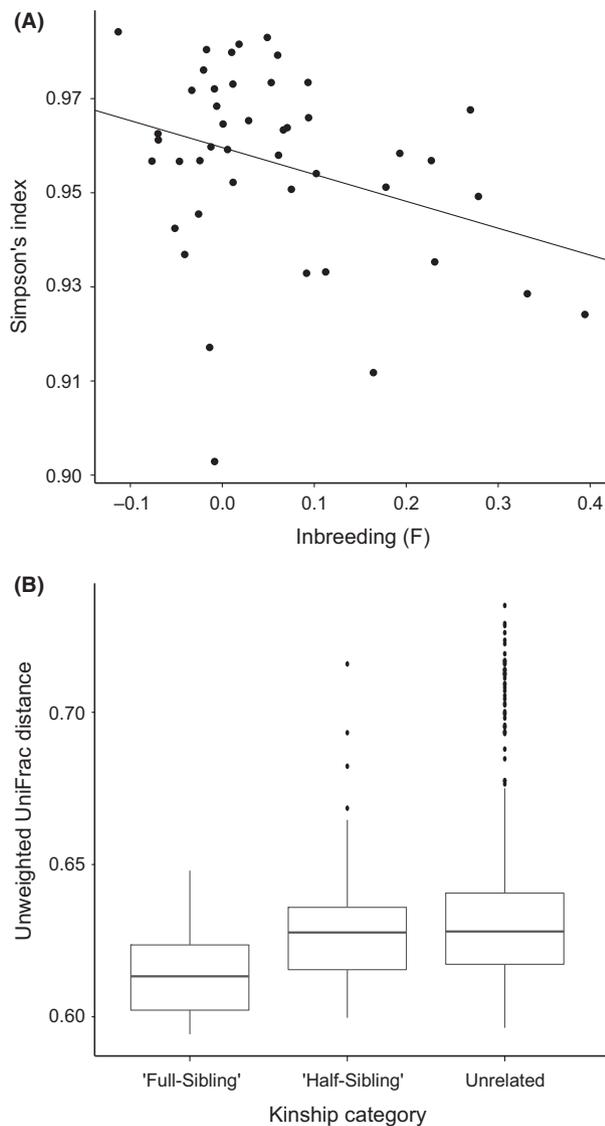


Fig. 4 (A) Scatterplot of inbreeding coefficients and Simpson's diversity index. (B) Box plot of pairwise UniFrac distances for each COLONY-based kinship category.

among management units overall ($P < 0.05$), applying *adonis* to only management unit pairs with homogenous variances (HE-HW, $P = 0.55$; HG-MD, $P = 0.39$) still recovers significant or marginally significant effects (HE-HW, $R^2 = 0.025$, $P = 0.08$; HG-MD, $R^2 = 0.037$, $P < 0.01$). We also found significantly overdispersed variances between age classes (PERMDISP: $P < 0.05$), but not between sexes ($P = 0.80$). Finally, the first unweighted UniFrac principal coordinate (permutation test: $R^2 = 0.684$, $P < 0.001$) and the first two weighted UniFrac principal coordinates (PCo1, $R^2 = 0.166$, $P = 0.010$; PCo2, $R^2 = 0.213$, $P = 0.003$) were significantly correlated with carapace length, and the first principal coordinate of weighted UniFrac distance is

Table 2 Genera that were significantly correlated with or differed across variables associated with gut microbial communities

Genus (family)	Pearson's rho	FDR <i>P</i> -value
Carapace length		
Unnamed (Unnamed Bacteroidales)	0.498	0.007
Unnamed (Unnamed RF39)	0.450	0.007
Unclassified (Clostridiaceae)	0.423	<0.001
<i>Clostridium</i> (Clostridiaceae)	0.439	0.011
Unclassified (Unclassified Bacteroidetes)	-0.378	0.081
Age class		
<i>Solibacillus</i> (Planococcaceae)	-	0.011
<i>Alistipes</i> (Rikenellaceae)	-	0.011
Unclassified (Planococcaceae)	-	0.011
Unclassified (Unclassified Rhizobiales)	-	0.023
Unit		
<i>Yersinia</i> (Enterobacteriaceae)	-	0.025
<i>SMB53</i> (Clostridiaceae)	-	0.037

Unclassified—did not match available GreenGenes sequences at a given taxonomic resolution.

Unnamed—matched to a GreenGenes reference sequence without available taxonomy.

Italicized *P*-values were marginally significant.

positively correlated with inbreeding ($R^2 = 0.092$, $P = 0.012$) (Fig. S1, Supporting information).

Discussion

Core microbiome of a hindgut-fermenting tortoise

The gut microbiome of *G. polyphemus* is highly diverse with 1470 core OTUs. The dominant phyla were Bacteroidetes and Firmicutes, consistent with results from other herbivorous reptiles and mammals (Ley *et al.* 2008; Pope *et al.* 2010; Hong *et al.* 2011; Delsuc *et al.* 2014; Gaillard 2014). The relative abundance of Firmicutes (36.0%) in our samples was much lower than those reported in other herbivorous reptiles, including the Galápagos tortoise (*Chelonoidis nigra*; 81.1%), green iguana (*Iguana iguana*; 74.0%), marine iguana (*Amblyrhynchus cristatus*; 75.1%), Galápagos land iguana (*Conolophus subcristatus*; 63.9%) (Hong *et al.* 2011) and northern gopher tortoise populations (59.7%; Gaillard 2014). In contrast, the relative abundance of Bacteroidetes (36.5%) was higher in our study population than in northern gopher tortoise populations and other reptiles (Gaillard 2014). The relative abundances of microbial phyla in gopher tortoise faeces were more similar to those reported in a diverse assemblage of hindgut-fermenting mammals, as might be expected

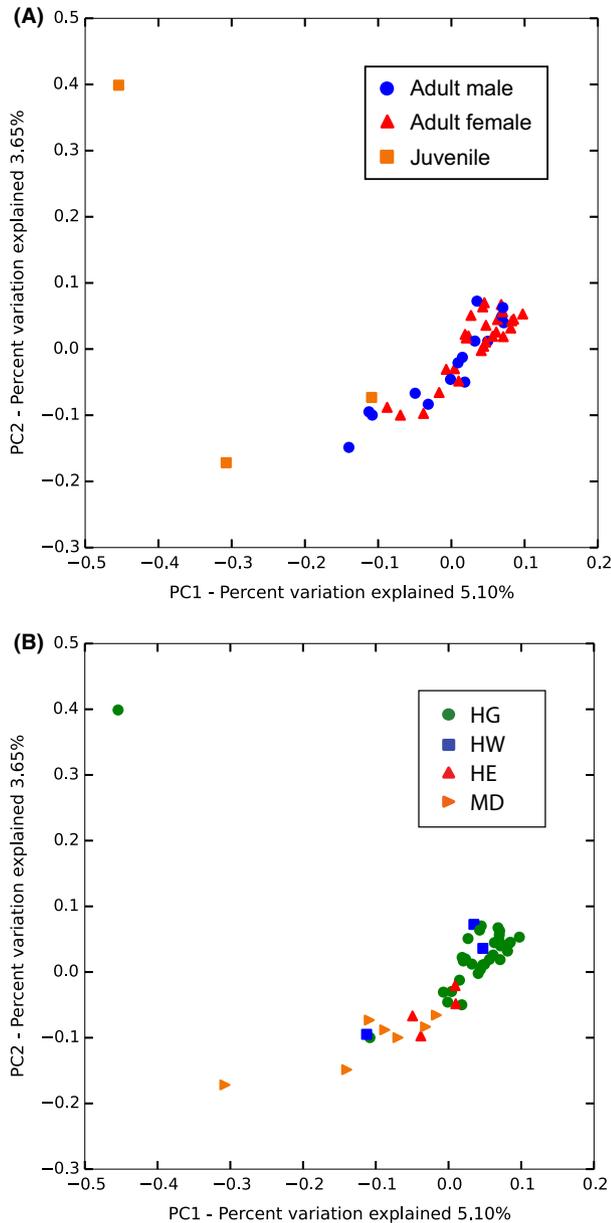


Fig. 5 Principal coordinate analysis (PCoA) plots for gopher tortoise gut microbial communities. Plots are based on unweighted UniFrac distances and are coded by (A) age class, sex and (B) collection unit.

based on similarities in diet and digestive morphology (i.e. hindgut fermentation) (Muegge *et al.* 2011; Delsuc *et al.* 2014).

Previous characterizations of reptilian gut communities have focused on tropical species, and hence, the greater relative abundance of Bacteroidetes in gopher tortoises could result from their subtropical environment. Because ectotherms cannot metabolically regulate internal body temperature, it has been suggested that herbivory in reptiles is constrained by the ability to

maintain high internal body temperatures to facilitate fermentation (King 1996; Mackie *et al.* 2004). Therefore, it is possible that, in smaller herbivorous reptiles or those living in cooler climates, alternative gut community states are necessary for efficient digestion. Climate and associated differences in behaviour and life history may also underlie differences between our south-central Florida study population and more temperate populations. Alternatively, because methodology can influence next-generation sequencing characterization of microbiota (Hamady & Knight 2009; Bahl *et al.* 2012; Maukonen *et al.* 2012; but see Lauber *et al.* 2010; Caporaso *et al.* 2012; Luo *et al.* 2012), it is possible that some of the observed differences are an artefact of methodology. Despite the observed differences, current surveys of reptilian gut microbiomes are still too sparse to convincingly draw broad conclusions about evolution and convergence across taxa; therefore, a more systematic analysis of variation across reptilian taxa is clearly warranted.

Firmicutes is the only phylum universally represented in mammalian faeces and is known to play important functions in digestion and host metabolism (Ley *et al.* 2008). Clostridia comprise the majority of Firmicutes in both reptilian and mammalian herbivores (Ley *et al.* 2008; Hong *et al.* 2011). In our population of gopher tortoises, >97% of all Firmicutes were clostridians. Several groups of Clostridia are capable of cellulose digestion (Ng *et al.* 1977; Ohara *et al.* 2000; Schwarz 2001; Nelson *et al.* 2003) and play major roles in herbivore digestion (Flint *et al.* 2008). We recovered core microbiome OTUs closely related to many of these Clostridia groups (e.g. Ruminococcaceae, Clostridiaceae, Lachnospiraceae), showing that this pattern extends to gopher tortoises. Members of the genera *Ruminococcus* and *Clostridium*, both containing known cellulolytic species (Ng *et al.* 1977; Desvaux *et al.* 2000; Ohara *et al.* 2000), constituted about 1.4% and 1.3% of the overall tortoise gut microbiome, respectively. The other dominant phylum in the gopher tortoise gut microbiome, Bacteroidetes, is metabolically diverse (Shah & Gharbia 1993), and its widespread dominance in vertebrate digestive organs (Ley *et al.* 2008; Hong *et al.* 2011) implies an important role in digestion and metabolic function. Members of the Bacteroidetes promote the initial digestion of simple and complex polysaccharides, including celluloses and hemicelluloses (Shah & Gharbia 1993).

As expected from other nonavian reptiles, we did not recover true Fibrobacteres (Hong *et al.* 2011), which are important cellulose digesters in herbivorous mammals (Ransom-Jones *et al.* 2012). However, we did recover members of the closely related candidate phylum Termitite Group 3 (TG3). Specifically, we recovered an

average 4% relative abundance of bacteria belonging to an unnamed genus within TG3 subphylum-1. TG3 bacteria are presumed to be cellulose-digesting in termite guts (Hongoh *et al.* 2006; Mikaelyan *et al.* 2014). While highly associated with Blattodea, members of the TG3 group have also been found in lakes and sediments (Hongoh *et al.* 2006). However, to our knowledge, members of TG3 have never been reported in vertebrates, even transiently in myrmecophagous mammals (Delsuc *et al.* 2014). The presence of TG3 was not mentioned in previous work on northern gopher tortoises; however, because a complete profile of the core faecal microbiome was not presented (Gaillard 2014), the group may have been overlooked or its absence may result from methodological or geographic differences. Although we cannot definitively rule out the possibility that TG3 sequences represent transient bacteria passing through the digestive tract, the presence of this lineage in all of our gopher tortoise samples may indicate a role in cellulose digestion and that TG3 lineages inhabit more taxonomically diverse hosts than previously recognized.

Members of the genus *Treponema* (Spirochaetes: Spirochaetaceae) are also relatively abundant (~6%) in gopher tortoises. *Treponema* cause several mammalian diseases and are largely absent from the gut communities of humans, although they have been observed as nonpathogenic gut community members in ruminants and nonhuman primates (Stanton & Canale-Parola 1979; McKenna *et al.* 2008). Although noncellulolytic, some *Treponema* species associate with specific plant substrates during digestion (Bekele *et al.* 2011) and have been shown to facilitate digestion of celluloses by co-occurring bacteria (Kudo *et al.* 1987). Thus, *Treponema* may also play an important role in the tortoise gut microbiome.

Archaea comprised about 1.1% of *G. polyphemus* faecal communities, which is consistent with the relative abundances of Archaea reported from mammalian ruminants (Ziemer *et al.* 2000). Archaea within gopher tortoise gut communities were all members of either the Methanobacteria group of known hydrogenotrophic methanogens (Balch *et al.* 1979; Boone *et al.* 1993) or the Thermoplasmata (i.e. *vadinCA11*), which are known to be methylotrophic methanogens (Poulsen *et al.* 2013) (Fig. 2). Methanogens appear to be ubiquitous across herbivorous vertebrates (Hackstein & van Alen 1996). Hydrogenotrophic methanogens facilitate formation of volatile fatty acids (VFAs) and remove inhibitors to bacterial fermentation of plant material (Latham & Wolin 1977; Janssen & Kirs 2008). Patterns of methane production scaled to body size are similar between tortoises and hindgut-fermenting mammals (Franz *et al.* 2011), supporting the convergence of gut microbiota between these groups.

Fine-scale spatial structure of faecal microbiota

Geography and habitat type are typically strong influencers of gut microbial community composition and structure (Fallani *et al.* 2010; Lankau *et al.* 2012; Linnenbrink *et al.* 2013), yet northern gopher tortoise populations do not exhibit gut community differences based on geographic distance or soil and habitat types (Gaillard 2014). Conversely, we detected fine-scale spatial structure, across far shorter geographic distances, based on fire management units (Fig. 5B). The influence of management unit, combined with the lack of geographic genetic structure among our samples, indicates that differences are likely driven by local dietary availability or environmental microbiota. However, the observed effects were weak, suggesting that the role of habitat as a determinant of tortoise gut microbiomes is relatively small. In northern gopher tortoise populations, differences in soil microbiota do not drive tortoise gut communities (Gaillard 2014). Similarly, fine-scale geographic structure in our population at Archbold was not associated with soil type or time since last fire (Table S1, Supporting information). However, historical land use and introduced forage species within HG may be shaping faecal microbial communities in tortoises associated with that management unit. Gopher tortoise diets largely consist of the dominant forage species in their habitats (MacDonald & Mushinsky 1988). Unlike other management units, HG is dominated by exotic grass (*P. notatum*), and thus may represent a special case of disturbance to enteric microbial communities due to anthropogenic habitat alteration.

The proximity of HW and HG might explain the clustering of some individuals across those units, particularly because some tortoises from HW were collected near HG and thus may feed in that management unit. Adult tortoises can easily travel among all sampled units and across the highway (B. B. Rothermel, personal observation). The distance between MD and HE is within the maximum dispersal recorded in gopher tortoises (Eubanks *et al.* 2003). Male dispersal to HG may explain the two individuals which cluster with tortoises from MD and HE based on similarities in gut microbiota (Fig. 5B). One of these males is known to live on the HG boundary, having previously been recorded in a management unit adjacent to HE. All females clustered with their respective management units, likely because they generally maintain smaller home ranges and exhibit greater site fidelity (Eubanks *et al.* 2003; Guyer *et al.* 2012). Our data demonstrate that structure in microbial communities can exist across short geographic distances and permeable landscapes.

Host genetic structure, inbreeding and microbial diversity

It has been hypothesized that host genetics influences microbial communities, yet the extent to which host genetics systematically controls microbiota remains unresolved (Spor *et al.* 2011). Specific host immune loci influence gut microbiota in sticklebacks (Bolnick *et al.* 2014a), and neutral variation in wild house mice is correlated with caecal mucosal community variation, but not caecal content community variation (Linnenbrink *et al.* 2013). We found no effect of host genetic background on gopher tortoise gut communities, suggesting a lack of host genetic influence on gut communities in this species. However, variation at neutral markers may not accurately reflect variation at potentially relevant genes, particularly those under selection.

Increased inbreeding was associated with shifts in relative abundances of gut microbes (Fig. 4) likely due to decreased dominance of Firmicutes and Bacteroidetes OTUs. Because all fire management units had similar levels of overall inbreeding (Table S1, Supporting information), the influence of inbreeding does not appear to be an artefact of spatial distribution of tortoises. Comparisons between inbred domestic and outbred wild *Mus musculus* have similarly revealed a lack of richness effects, but have detected changes in relative abundance due to inbreeding (Kreisinger *et al.* 2014). Although our observed effect sizes were small, it is possible that minor changes in functionally important gut microbiota could still meaningfully impact host organisms. Because we used neutral markers to estimate genetic diversity, we cannot determine whether these shifts in microbial community abundance have any functional or fitness implications for tortoises. However, inbreeding is often correlated with decreased population fitness (Reed & Frankham 2003). Given the importance of mutualistic gut microbes in proper metabolism, digestion and pathogen defence (Flint *et al.* 2008; Li *et al.* 2008; Stecher & Hardt 2011), changes in microbial community composition with host genetic diversity warrant further investigation in this and other threatened species.

Role of kinship and coprophagy in microbial transmission

We detected an effect of kinship on gut community diversity and composition. Kinship plays an important role in shaping microbial communities in several species including humans (Funkhouser & Bordenstein 2013) and green iguanas (Troyer 1982, 1984b). However, the influence of kinship in gopher tortoises appears to be restricted to highly related individuals such as sibling or parent–offspring pairs (Fig. 4). The restriction of

this effect to dyads with the equivalent to ‘full-sibling’ relatedness, in combination with low overall variation, may have limited our capacity to detect this effect using the less powerful mantel tests. The lack of a host genetic cluster effect on microbiota suggests that the observed similarity among close kin is driven by filial transmission of microbes rather than heritable genetic control of gut communities. In species with parental care, the influence of relatedness may result from continued association of individuals in kin groups (Troyer 1984a; Lombardo 2008). However, gopher tortoises lack parental care, and thus, kinship effects could be explained by three nonexclusive mechanisms: direct parental transmission during egg development, sibling association in the nest prior to emergence, or biased coprophagy towards close kin. Independent of the exact mechanism, our results indicate that microbiota effects occur only among close kin.

Although coprophagy may be important in the transmission of gut microbiota among gopher tortoises, we cannot demonstrate that kin-directed coprophagy underlies the observed patterns. Given that coprophagy in desert tortoises, *G. agassizii* and *G. morafkai*, has only been documented in hatchlings (Lance & Morafka 2001) and that only a single inoculation is necessary in green iguanas (Troyer 1984b), any influence of coprophagy on gut microbiota is likely determined early in life. Further work focusing on tortoise social networks and hatchling tortoises with known mothers is needed to better understand gut community transmission and development. Because they are endangered, member of the genus *Gopherus* is often the focus of head-starting programmes (Edwards *et al.* 2014; Tuberville *et al.* in press), offering a resource for future studies to comprehensively investigate gut microbiome dynamics and transmission in neonates.

Potential ontogenetic community shifts

Our findings provide some evidence that microbial community structure and composition may undergo changes from juvenile to adult hosts. Comparisons of alpha diversity indicate that differences between age classes are due predominately to increasing richness and shifts in rare taxa, a pattern similar to that observed in other vertebrates (Hopkins *et al.* 2002; Funkhouser & Bordenstein 2013). These shifts in gut communities could potentially reflect ontogenetic changes in dietary habits. Juvenile gopher tortoises consume higher protein diets and comparably less grass (Poaceae) than adults (MacDonald & Mushinsky 1988). However, our results should be interpreted with caution, because our sampling of juvenile tortoises was limited to three individuals due to the naturally low

encounter rate of this life stage. Adult gut microbial communities in our population were relatively homogeneous (Fig. 2) and the effects of carapace length were not detected when juvenile tortoises were removed. Further studies with increased sample size or directly observing changes in gut communities across individual lifespans are necessary to confirm this pattern. Acquiring this kind of longitudinal data as part of long-term monitoring of natural populations would be a fruitful avenue of research.

Microbiome homogeneity across individuals

Overall, adult faecal-associated microbial communities were relatively homogeneous in tortoises (Fig. 2) compared to other vertebrates (Lozupone *et al.* 2012; Nelson *et al.* 2013; Bolnick *et al.* 2014b). High gut microbiome homogeneity across individuals combined with a diverse assemblage of OTUs may have limited our ability to detect effects at the OTU level. Across-site variation in gopher tortoise gut microbial communities throughout their range is similarly limited (Gaillard 2014). Greater convergence of mammalian gut communities has been observed in species with more specialized diets such as herbivory and myrmecophagy, potentially due to constraints of microbe-mediated cellulose or chitin digestion, compared with generalist species (Ley *et al.* 2008; Delsuc *et al.* 2014). We propose that the observed homogeneity of gut communities in gopher tortoises is potentially due to strong functional constraints associated with herbivory in natural populations. Whereas species with more flexible diets may exhibit wider variance in gut communities even in natural populations (Nelson *et al.* 2013; Bolnick *et al.* 2014b,c), obligate herbivores are likely to be more reliant than other species on stable, specifically functioning gut communities.

Studies characterizing reptilian gut microbiota are sparse (Costello *et al.* 2010; Hong *et al.* 2011; Keenan *et al.* 2013; Gaillard 2014) despite the wide-ranging dietary modes and repeated independent evolution of herbivory (Vitt 2004). Thus, further work on a diverse assemblage of reptilian species across a variety of dietary modes is needed to understand the evolution of mutualistic gut microbes in this group. Our results provide further insight into the gut microbiota of herbivorous reptiles and evidence for both high levels of homogeneity across individuals and a role of fine-scale spatial distribution, inbreeding, kinship and potentially ontogeny in shaping the gut microbiota of gopher tortoises.

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K.R.Z. and M.L.Y. designed the study with contributions from coauthors. T.D.T. provided technical training and contributed to microsatellite optimization. S.H.D., B.B.R. and M.L.Y. conducted field work. M.L.Y. and A.V.L. collected genetic data and performed data analysis. M.L.Y. wrote the manuscript with important contributions from all coauthors.

Data accessibility

Microsatellite genotypes, GPS, map files, OTU tables and raw 16S rRNA sequence files used in QIIME are archived in Dryad doi:10.5061/dryad.54bm8.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Characteristics of each fire management unit sampled for this study including number of tortoises sampled (N), dominant habitat, soil type, last fire, mean inbreeding (F), mean relatedness (r), mean richness, mean phylogenetic diversity

(PD), and mean Simpson index. Standard deviations are given for alpha diversity metrics.

Table S2 Microsatellite markers used in this study. Number of alleles (k), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of identity and exclusion, annealing temperature, and primer source are noted

Table S3 Families that were significantly correlated with variables associated with gut microbial communities. No families were significantly different between categorical variables (Unit, Age Class). Italicized P -values were marginally significant.

Table S4 Summary information for each sampled gopher tortoise included in this study, including age class (J = juvenile, A = adult), carapace length (mm), sex, and locality (HE = Hill-East, HG = Hill Garden, HW = Hill-West, MD = Main Drive). Genetic clusters were only designated for samples assigned to a genetic cluster with a membership coefficient ≥ 0.75 . GPS coordinates are provided in the Universal Transverse Mercator (UTM) coordinate system for UTM zone 17N.

Fig. S1 Scatterplots of continuous variables (carapace length and inbreeding coefficient) and the first two principle coordinates (PCo) partitioned from either the weighted or unweighted UniFrac distance metric. Best-fit-lines are solid for PCo1 and dashed for PCo2. (A) Carapace length and unweighted UniFrac, (B) Carapace Length and weighted UniFrac, (C) inbreeding and unweighted UniFrac, and (D) inbreeding and weighted UniFrac.

Fig. S2 Heatmap displaying relative abundances of genera for each individual. Juvenile samples are presented separately, with adult individuals grouped by management unit. Numbers represent relative abundances for each cell as a proportion. Proportions do not sum to 100 due to the removal of genera with relative abundances below 1%.

Fig. S3 UPGMA tree based on unweighted UniFrac distances. Each tip is coded by sex, management unit, and genetic cluster, if assigned.