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The Reptile Gut Microbiome: Its Role In Host Evolution And Community Assembly

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THE REPTILE GUT MICROBIOME: ITS ROLE IN HOST EVOLUTION AND COMMUNITY ASSEMBLY

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ABSTRACT

I characterize the endogenous (gut) microbiome of Squamate reptiles, with a particular focus on the suborder Serpentes, and investigate the influence of the microbiome on host evolution and community assembly using samples I collected across three continents in the New and Old World. I developed novel methods for sampling the microbiomes of reptiles and summarized the current literature on non-mammalian gut microbiomes. In addition to establishing a standardized method of collecting and characterizing reptile microbiomes I made novel contributions to the future direction of the burgeoning field of host-associated microbiome research. Through persistent and rigorous fieldwork I amassed the largest dataset of non-mammalian vertebrate microbiomes in existence. By incorporating emergent next generation sequencing technologies and combining cross-disciplinary methods from the fields of phylogenetics and community ecology, I show that the core reptile gut microbiome is comprised of members of the Proteobacteria, Firmicutes and Bacteroidetes; and that reptile gut bacterial communities are more similar to those of birds than mammals—a previously untested hypothesis. I show that the reptile gut microbiome is strongly influenced by host phylogeny and several ecological traits including parity and foraging mode. My analyses reveal that the composition of the reptile gut microbiome is influenced by *who* (phylogeny)*, where* (geographic locality) a host organism is and *how* she lives. Broadly, this work reflects that the microbiome of reptiles is both a phylogenetic and ecological trait that is influenced by selection and that host-associated microbiomes harbor a wealth of natural history information waiting to be explored.

DEDICATION

I dedicate this work to my wife, Dr. Katya Colston,

and eldest daughter, Ms. Maddison Colston,

for their unceasing love, dedicated and unwavering support and encouragement

during the pursuit of my academic goals and lengthy (often years) of absence abroad to collect

data.

ACKNOWLEDGEMENTS

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CHAPTER I

MICROBIOME EVOLUTION ALONG DIVERGENT BRANCHES OF THE VERTEBRATE TREE OF LIFE: WHAT'S KNOWN AND UNKNOWN

Colston, T.J. & Jackson, C.R. (2016) *Molecular Ecology* **25**: 3776–3800.

ABSTRACT

Vertebrates harbor microbes both internally and externally, and collectively these microorganisms (the "microbiome") contain genes that outnumber the host's genetic information ten-fold. The majority of the microorganisms associated with vertebrates are found within the gut; where they influence host physiology, immunity, and development. The development of next generation sequencing has led to a surge in effort to characterize the microbiomes of various vertebrate hosts, a necessary first step to determine the functional role these communities play in host evolution or ecology. This shift away from a culture-based microbiological approach, limited in taxonomic breadth, has resulted in the emergence of patterns suggesting a core vertebrate microbiome dominated by members of the bacterial phyla Bacteroidetes, Proteobacteria and Firmicutes. Still, there is substantial variation in the methodology used to characterize the microbiome, from differences in sample type to issues of sampling captive or wild hosts; and the majority (>90%) of studies have characterized the microbiome of mammals, which represent just 8% of described vertebrate species. Here, we review the state of microbiome studies of non-mammalian vertebrates and provide a synthesis of emerging patterns in the microbiome of those organisms. We highlight the importance of collection methods, and the need for greater taxonomic sampling of natural rather than captive hosts; a shift in approach that is needed to draw ecologically and evolutionarily relevant inferences. Finally, we recommend future directions for vertebrate microbiome research, so that attempts can be made to determine the role that microbial communities play in vertebrate biology and evolution.

INTRODUCTION

Microorganisms, primarily bacteria, can be found living both on and in all animals. It has generally been thought that the number of bacterial cells associated with an animal exceeds the number of the host animal's cells at least tenfold (Savage 1977; Berg 1996), although newer estimates suggest that this ratio may be more in the range of 1:1 (Rosner 2014; Sender *et al.* 2016). Regardless of the total number of microbial cells, collectively, the genomes of these microorganisms may contain 10-100 times as many genes as the host's genome (Berg 1996; Savage 1977;but see Rosner 2014; Sender *et al.* 2016). These microbes aid in the host's nutrient acquisition and immune response, and can influence host behavior, development, reproduction and overall health (Fraune & Bosch 2010; Colombo *et al.* 2015). The influence of the host on their microbiome is still being determined, but both host diet and phylogeny have been shown to be important predictors of endogenous (gut) microbial community composition (Ley *et al.* 2009; Sanders *et al.* 2013; Clements *et al.* 2014; Mikaelyan *et al.* 2015). Much of this information is derived from culture-independent (i.e. molecular) studies, which have primarily sequenced fragments of the bacterial 16S rRNA gene. The development of next-generation sequencing (NGS) technologies over the last decade has greatly facilitated such studies, allowing both rapid and affordable sequencing at the depth needed to sufficiently characterize diverse bacterial communities (Turnbaugh *et al.* 2007; Gloor *et al.* 2010; Arumugam *et al.* 2011a; Bartram *et al.* 2011).

The apparent relationship between host phylogeny (or genotype) and microbial community composition has led to much discussion of co-evolution of microbial communities and their multicellular hosts (Ochman *et al.* 2010; Anderson *et al.* 2012; Phillips *et al.* 2012b; Moeller & Ochman 2014). However the vast majority of work on gut microbial communities has

focused on mammals, particularly humans (Ley *et al.* 2006, 2008, 2009; Arumugam *et al.* 2011b; Yatsunenko *et al.* 2012). Furthermore, the majority of non-human mammalian microbiome studies have tended to characterize fecal microbiomes from captive animals, often from laboratories or zoos (Ley *et al.* 2009a; b & refs within). Given that we know that human gut microbiomes are largely developed at an early age and are related to both the diet and environmental conditions of the individual host (Koenig *et al.* 2011; Lozupone *et al.* 2012), it is questionable whether work on captive animals can be used to predict the gut microbiomes of animals in the wild. This problem has been suggested before (Amato 2013), yet there is still a substantial lack of studies that have attempted to characterize enteric microbial communities in hosts within a natural environment (Table 1, Table S1 Appendix). This lack of knowledge becomes even more pronounced when we extend the focus beyond mammals to other vertebrates, with the gut microbial communities of major branches of the vertebrate tree of life such as amphibians, reptiles, birds, and fish being very poorly described (Figure 1).

Table 1. Summary of host organism, technology used.
Next generation sequencing (NGS), traditional molecular methods (TMM; including Sanger sequencing, DGGE, TGGE, PCR, qPCR) culture or microscopy (including florescent imagery and SEM), wild vs. captive, and number of publications from 229 published studies since 1990. A more detailed table which further categorizes the studies according to both technology used, sample type and genetic marker sequenced is available in the Appendix Table S1.

Organism	Technology used	Captive (C) or wild (W) host	Number of published studies
Fish	NGS	C	19
Fish	NGS	W	3
Fish	NGS	W&C	2
Fish	Cloning & TMM	C	2
Fish	Cloning & TMM	W	4
Fish	Culture & TMM	C	35
Fish	Culture & TMM	W	6
Fish	Culture & TMM	W&C	1
Fish	Culture only	C	23

Fish	Culture only	W	8
Fish	Culture only	W&C	1
Fish	Culture, cloning, TMM	W	1
Fish	Cloning, microscopy & TMM	C	2
Fish	TMM	C	22
Fish	TMM	W&C	1
Fish	TMM & NGS	C	2
Fish	Microscopy	C	4
Fish	Microscopy	W	3
Frog	NGS	C	2
Frog	NGS	W	3
Frog	NGS	W&C	2
Frog	Culture NGS	W	1
Frog	Culture & TMM	C	3
Frog	Culture & TMM	W	7
Frog	Culture & TMM	W&C	1
Frog	Culture only	C	6
Frog	Culture only	W	5
Frog	TMM	C	1
Frog	TMM	W&C	2
Frog (tadpole)	NGS	C	1
Salamander	NGS	W	4
Salamander	NGS	W&C	2
Salamander	Culture & NGS	W	1
Salamander	Culture & TMM	C	1
Salamander	Culture & TMM	W	4
Salamander	Culture only	W	1
Salamander	TMM	C	1
Salamander	TMM	W&C	1
Lizard	NGS	C	1
Lizard	NGS	W	1
Lizard	TMM	C	1
Snake	NGS	C	1
Snake	NGS	W	1
Snake	Cloning & TMM, NGS	W	1
Snake	Culture only	W	1
Snake	TMM	C	1
Snake	TMM	W	2

Tortoise	NGS	W	1
Alligator	NGS	W&C	2
Bird	NGS	C	2
Bird	NGS	W	8
Bird	NGS	W&C	4
Bird	Cloning and TMM	W	11
Bird	Culture only	W	1
Bird	Culture only	W&C	1
Bird	PCR	W&C	1
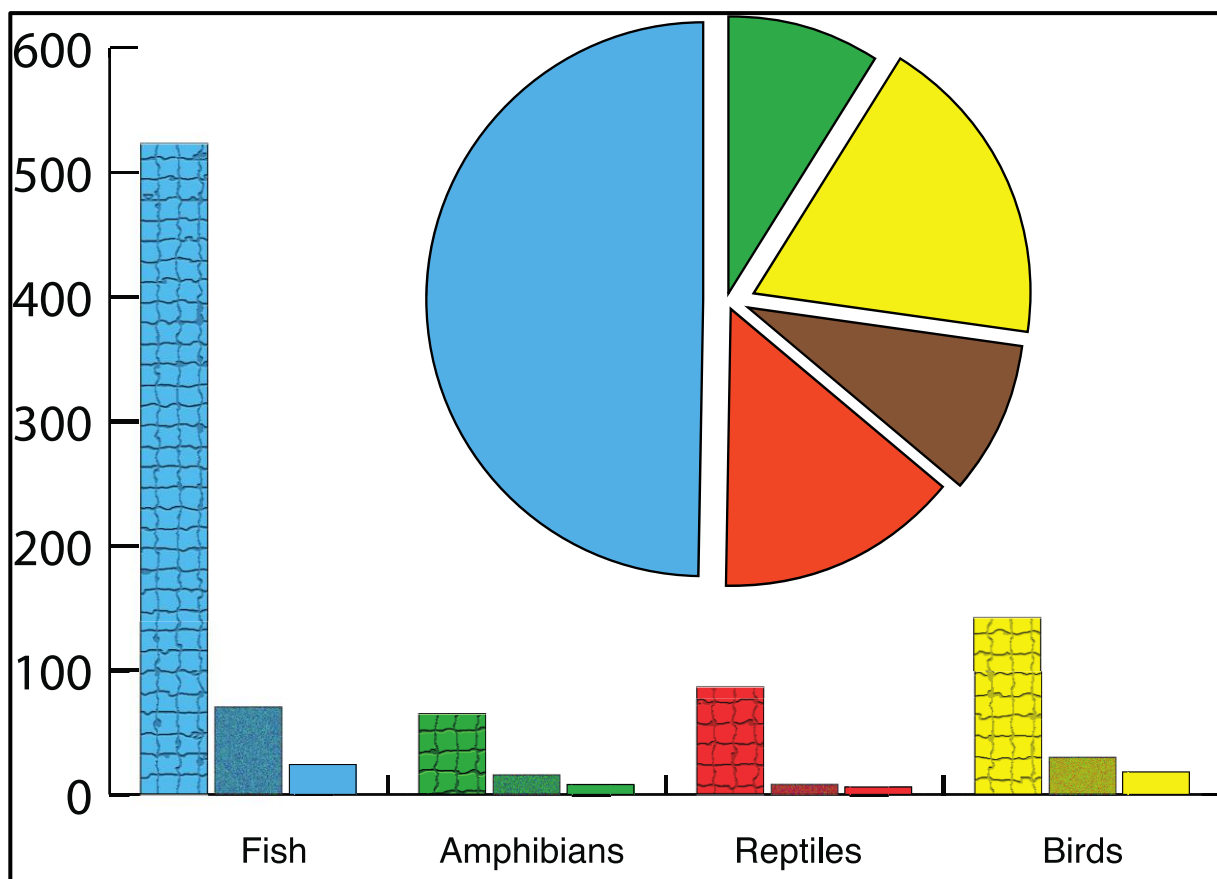
Figure 1. Distribution of microbiome studies across vertebrates.
Column chart describing the number of families (tiled bar), number of families whose microbiome has been studied (hatched bar), and number of families whose microbiome has been investigated using NGS methods (plain bar) for fish (blue), amphibians (green), reptiles (red) and birds (yellow). Inset pie chart displays percentage of vertebrate species described and includes mammals (brown).

The microbiome has been linked to changes in host growth rate and metabolism (De Winter *et al.* 2015), host phylogeny (Anderson *et al.* 2012; Phillips *et al.* 2012a; Colman *et al.* 2012), host ecology and life history (Wong & Rawls 2012; Coon *et al.* 2014; Dill-McFarland *et al.* 2014) and geography (Hird *et al.* 2014). These emerging patterns have led to the hypothesis that endogenous microbiomes reflect the evolutionary signatures of their hosts, and that ecological and evolutionary forces act on both the host and its resident microbiome. Microbes may in turn affect the evolution of the host, and microbes may have influenced vertebrate host evolution for millions of years, potentially contributing to the evolutionary trajectories of entire vertebrate communities (Zilber-Rosenberg & Rosenberg 2008; Ley *et al.* 2009; Fraune & Bosch 2010). Ecological studies stand to gain much by incorporating knowledge of the host organism's microbiome, and microbiome research can fundamentally change how we approach questions in evolutionary biology. As we assess the existing knowledge of non-mammalian vertebrate microbiomes, with particular attention to the endogenous (gut) microbiome in the context of better-known mammalian (i.e. human and primate) taxa, we highlight questions that remain unaddressed in these systems and make recommendations for future avenues of research in light of rapidly advancing sequencing technologies.

PATTERNS IN THE MICROBIOME ALONG THE VERTEBRATE TREE OF LIFE

Fishes

Fishes are the most diverse group of vertebrates with nearly 34, 000 described species as of early 2016, and ray-finned fish encompass half of all known vertebrate species (fishbase.org). Fish are anamniotic ecototherms that require aquatic habitat for survival. One of the most successful vertebrate groups, fish occupy marine and freshwater habitats across the globe, and have adapted to live in some of the most extreme environments of any vertebrate (e.g. some species tolerate hydrogen sulfide streams). Fish have a variety of reproductive strategies and in terms of diet may be herbivorous, omnivorous or carnivorous (Nelson 2006), suggesting that the microbiomes of fish could be highly variable, depending upon both host phylogeny and environmental conditions.

The microbiomes of fish are among the better characterized of non-mammalian vertebrates, likely because of the greater importance of fish as a food resource or for recreational activity. As with studies of the microbiota of other host taxa, much of the work on the autochthonous microbial communities of fish has been largely culture-based, with culture-independent methods being used only recently. Increased ease of analysis from advancing technologies such as NGS, coupled with the importance of fish in aquaculture and their breadth of ecologies, has meant that the microbiomes of fish have received much attention in recent years, and patterns in fish microbiome structure has previously been reviewed (Clements *et al.* 2014).

Exogenous microbiomes of fishes

The mucosal and skin microbiomes of fishes has been less studied than that of amphibians, but more so than that of reptiles including birds. Mucosal microbiota appear to be

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important in host physiology, and help with the development of adaptive immunity in mammals, particularly humans (Human Microbiome Project Consortium 2012), although this relationship is less understood in other vertebrates. The transition from aquatic to terrestrial life over the course of vertebrate evolution also provided opportunity for adaptive shifts in mucosal microbiomes (Lowrey *et al.* 2015), so that examining the mucosal or cutaneous microbiomes of fish could be critically important in assessing these shifts. NGS and targeted bacterial 16S rRNA gene scans were used to assess the bacterial diversity of five mucosal surfaces of captivity-raised rainbow trout (*Oncorhynchus mykiss*), revealing that the Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Tenericutes were the dominant bacterial phyla, and that the skin had the most diverse bacterial communities of any surface investigated (Lowrey *et al.* 2015). *Flectobacillus* and *Flavobacterium* were the dominant bacterial genera found both on the skin and gills, but the proportions of these genera were variable and they comprised 3.5-35% of the total community in different samples. External mucosal surfaces shared the most similar microbial communities, but all five mucosal surfaces examined (both internal and external) were shown to have distinct "core" microbial communities (Lowrey *et al.* 2015). Mucosa were also screened for the presence of known fungal pathogens to amphibians and fish as well as for the presence of anti-fungal properties in the microbiome; 28% of the identifiable bacterial operational taxonomic units (OTUs; a surrogate for bacterial species based on sequence similarity) matched with cultivable, fungal-resistant bacteria known from amphibian skin (Lowrey *et al.* 2015). While this study had limited sample size (just six individuals), and used captive-reared rather than wild hosts, it provides the first thorough picture of a teleost fish microbiome, and suggests that there may be some overlap between the cutaneous microbiomes of fish and amphibians.

While some studies have suggested that the skin and mucosal microbiomes of freshwater fish show lower bacterial diversity than gut communities (Boutin *et al.* 2014; Leonard *et al.* 2014), high bacterial diversity has been found on the skin of killifish (*Fundulus grandis*) (Larsen *et al.* 2015), as well as in the afore-mentioned study of rainbow trout (Lowrey *et al.* 2015). For aquatic organisms, constantly exposed to bacteria in the medium that they inhabit, this finding would not be surprising as the microbiome of the aquatic environment itself is generally more diverse than the skin surfaces of other aquatic organisms (Apprill *et al.* 2014; Bik *et al.* 2016). Thus, the cutaneous microbiome of fish may share some properties with that of larval amphibians (Kueneman *et al.* 2014), in that it may be influenced by both the host and the bacterial composition of the surrounding water. It would be interesting to compare the skin microbiomes of different organisms inhabiting the same aquatic environment (e.g. a shared pond) to see if there is overlap in the microbiomes of coexisting aquatic vertebrates, potentially a result of each being influenced by the same microbial community in the water column or sediment.

Comparisons between the skin and gut microbiomes of fish are more limited, although culture-dependent approaches have suggested that the skin microbiome of captive reared catfish (*Clarias gariepinus*) has similar levels of culturable diversity to that of the gut microbiome (Olojo *et al.* 2012). Temporal variability in the microbiome may also be important, and methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) analysis of 16S rRNA genes have suggested seasonal shifts in skin microbiome structure observed in both wild (Wilson *et al.* 2008) and captive fishes (Le Nguyen *et al.* 2008). In an effort to characterize the role of host specificity in the skin microbiome, over 100 individuals representing six fish species from four families were sampled in the Gulf of

Mexico and their skin microbiomes analyzed by ribosomal intergenic spacer analysis (RISA) and 16S rRNA gene NGS (Larsen *et al.* 2013). Sequencing revealed that Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the dominant bacterial phyla, with each fish species containing species-specific skin bacterial communities (Larsen *et al.* 2013). RISA results were less clear, however, and suggested a statistically significant effect of sampling date and individual, as well as host species. Overall, the data suggested a pattern of association in microbiome composition with sample date and locality, but also a strong influence of host species specificity. This supports the hypothesis that skin microbiomes of aquatic vertebrates are comprised of bacteria present in the surrounding environment (Wilson *et al.* 2008), but that those that are able to actually colonize and establish on a host show a phylogenetic component (Bik *et al.* 2016).

Endogenous microbiomes of fishes

Host physiology (McDonald *et al.* 2012), phylogeny, and ecology (Wong & Rawls 2012) have all been implicated in structuring the gut microbiota in fishes (reviewed in Clements *et al.* 2014). Time of day, seasonality and active digestion has also been shown to affect fish gut microbiota (Kohl *et al.* 2014; Fortes-Silva *et al.* 2015) suggesting a complex community impacted by multiple environmental variables. Clements *et al.* (2014) highlight the need to accurately classify host ecology (i.e. diet) in these analyses, in order to truly determine the functional roles of fish gut microbial communities, and some studies may have misclassified diet, drawing inaccurate conclusions on the role of the microbiome (Sullam *et al.* 2012). However, both culture-dependent studies and those based on DNA sequencing have generally confirmed the importance of members of the Proteobacteria and Tenericutes (Clements *et al.* 2014; Givens *et al.* 2015). Since 2014, >30 studies have been published that examined the gut

microbiomes of fishes using NGS techniques (Figure 2), and these data require a re-evaluation of

what we know about the endogenous microbial community of fish.
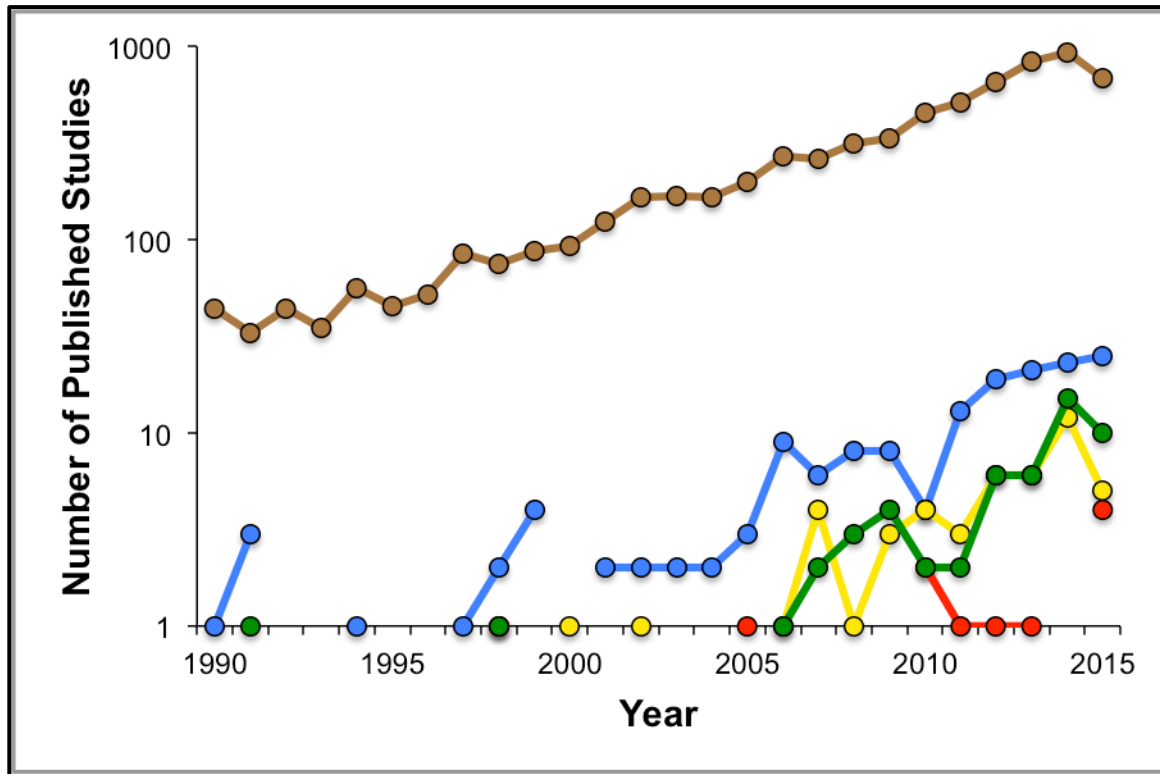
Figure 2. Increase in the number of microbiome studies for different classes of vertebrates over a 25 year period from 1990 to 2015.
Numbers were derived from a search of the Scopus database (www.scopus.com) for each of amphibians (green), birds (yellow), fish (blue), reptiles (red), and mammals (brown) using the search terms "microbiome" and/or "bacteria" and "gut", and not "mouth" and not "blood". Mammals included humans while for birds we excluded strictly domesticated poultry studies focused on pathogens. Because of an increased interest in that area, amphibians included the search term "skin". Publication number is shown on a log scale. Microbiome studies of mammals have increased at a fairly consistent exponential rate each year, whereas increases in studies of non-mammalian vertebrates have been more erratic and even combined fall substantially below those of mammals.

Surprisingly, given the potential for fish to acquire microbial populations from the

surrounding water, it has yet to become standard practice to compare the microbial composition

of the water that fish inhabit to that of their gut microbiome. Marine mammals harbor gut

microbial communities consisting of species found in seawater but forming distinct assemblages specific to the host species (Bik *et al.* 2016). It would be expected that gut microbial populations of fishes are also acquired from the environment (particularly for omnivorous or herbivorous fish species). Indeed, the gut bacterial communities of omnivorous fish have been shown to cluster with free-living aquatic bacterial communities rather than with the gut communities of herbivorous mammals, whereas the gut bacterial communities of carnivorous fishes cluster with those of carnivorous mammals (Sullam *et al.* 2012). Similarly, when fishes of different species, reflecting highly divergent phylogenetic positions and ecologies, are raised together in experimental ponds, they tend to have highly similar gut bacterial communities (Larsen *et al.* 2014), suggesting a strong influence of the local environment on the gut microbiome. That study found that the gut communities of three commercial fish species (channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus*) were dominated by Fusobacteria, and that just 11 bacterial genera, shared between the three species, made up approximately 98% of the bacterial sequences recovered from all samples. The gut microbiome of each of the three host species showed similar levels of bacterial species richness, but evenness was significantly lower in largemouth bass, potentially a reflection of this species' trophic status as a top predator (Larsen *et al.* 2014).

Commercially important fish species dominate studies on the fish gut microbiome. These studies have revealed interesting data that indicate fish gut microbiomes are not only structured according to dietary type and environmental conditions, but are significantly influenced by first feeding (Ingerslev *et al.* 2014), metabolic activity (Ni *et al.* 2014) and starvation (Xia *et al.* 2014). Interestingly, the gut microbiomes of a commercial trout species (*Oncorhynchus mykiss*) were found to be dominated by Firmicutes rather than Proteobacteria when juveniles were first

fed plant-based rather than marine-based food; and this effect was still seen after individuals were switched to marine-based food only, indicating the importance of first colonization effects in structuring the gut microbiome into adulthood (Ingerslev *et al.* 2014).

The Trinidadian guppy (*Poecilia reticulata*), a species that exists as two predation dependent ecotypes that differ in diet, morphology, life history and physiology, has been used as a model system to investigate the role of host life history on gut microbial community composition (Sullam *et al.* 2015). Despite differences in gut physiology and diet between the two ecotypes, gut microbial composition did not seem to be affected by ecotype in the wild guppies, which showed significant structuring in their gut microbial communities based on their stream of origin, as well as a temporal effect (Sullam *et al.* 2015). In contrast, captive guppies showed distinct gut microbial communities based on fish ecotype regardless of diet over the course of the experiment. Both wild and captive guppies had distinct core microbial communities that were different from one another, as well as from environmental water and sediment samples (Sullam *et al.* 2015). This suggests that the ecotype of wild guppies does not present a strong enough selection pressure to override the effects of locality or host genetics on the gut microbiome, but that the same is not true for guppies raised in captivity, an interesting finding as it highlights the care that needs to be taken when making inferences about the microbiome from captive animals. However, the dominant bacterial phyla in the guts of both wild and captive guppies, regardless of ecotype, were Tenericutes, Spirochaetes, Proteobacteria and Fusobacteria (Sullam *et al.* 2015). At a finer resolution, the host populations with the most dissimilar gut microbial communities were those that were most distant genetically, suggesting the influence of host evolutionary history (regardless of ecology) in the gut microbiome (Sullam *et al.* 2015).

Members of the Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria were detected in the gut microbiomes of 15 different fish species (12 bony fish, 3 sharks), and although the proportions of each phylum varied by both fish species and individual, Proteobacteria tended to dominate (Givens *et al.* 2015). Despite a high amount of variation at the individual level (likely a result of limited sample size), each of the fishes had a distinct microbial community and the three shark species contained gut microbiomes that were highly similar (69-97% shared OTUs; Givens *et al.* 2015). There were also significant differences in the gut microbial communities of wild vs cultured mummichogs (*Fundulus heteroclitus*) and between life stages (juvenile, intermediate and adult) of pinfish (*Lagodon rhomboides*), suggesting influences of diet, age, and the environment on the fish gut microbiome. Interestingly, no single OTU was shared across all 15 host species, but several core OTUs were present in multiple host species (i.e. with the three shark species; Givens *et al.* 2015).

The influence of growth rate on the fish gut microbiome has been investigated in killifish (*Kryptolebias marmoratus*) and Atlantic cod (*Gadus morhua*; Forberg *et al.* 2016). DGGE and 16S rRNA gene sequencing revealed that genetically identical killifish of the same age but of different size (i.e. therefore at different growth rates) had significant differences in the richness of their gut bacterial community, and clustering analyses separated the communities of fish with a large body size from those of fish with a small body size, albeit not significantly so (Forberg *et al.* 2016). The gut communities of small and large Atlantic cod (again, of the same age, but in this case genetically heterogeneous) were also significantly different in bacterial richness, and host size (growth rate) was significantly correlated with microbial composition. That the two species differed in their genetic diversity suggested the strong influence of host genetics, which was at least partially influencing growth rate, on the initial establishment of the fish gut

microbial community (Forberg *et al.* 2016). Laboratory studies on the model organism zebrafish (*Danio rerio)* have shown that both the composition and population size of the initial bacterial colonizers can affect subsequent colonization ability in the fish gut (Stephens *et al.* 2015), so that host genetic variability could have important implications for the microbiome in later life. The influence of genetic variation at the individual level also highlights the need for increased numbers of individuals to be sampled during microbiome studies, as much of our knowledge of fish microbiomes, and vertebrate microbiomes in general, is often based on just a few individuals that may not necessarily be representative of the genetic diversity within wild populations.

An example of a study that did sample extensively at the individual level is that of Llewellyn *et al.* (2015), who sampled the gut bacterial communities of 96 wild Atlantic salmon (*Salmo salar*) across their range in both freshwater and marine life stages, in order to examine biogeographic patterns. NGS of bacterial 16S rRNA genes revealed that individual salmon in their freshwater stage have similar gut microbial communities, regardless of locality, whereas dramatic differences in gut microbiome composition were detected between salmon in marine and freshwater stages, with adults retaining much of their marine microbiome when they reenter freshwater to spawn (Llewellyn *et al.* 2015). Proteobacteria and Tenericutes (particularly *Mycoplasma* sp.) were the dominant bacterial phyla in marine adults, whose gut microbiomes were generally characterized by low bacterial diversity and high inter-individual variability compared with those of juveniles; possibly indicative of both seasonality and dietary complexity (Llewellyn *et al.* 2015). Clearly migratory fishes can undergo drastic changes to their gut microbial community composition during ontogeny, but those changes do not appear to be a reflection of purely environmental factors. This supports the hypothesis that while the gut microbiomes of fish are more similar to the microbiome of their environments than those of

mammals, both phylogenetic factors and host ecology interact to structure the fish endogenous microbial community (Ghanbari *et al.* 2015).

Amphibians

Amphibians (frogs, salamanders & caecilians) are ectothermic, anamniotic vertebrates which occupy terrestrial, arboreal, fossorial and freshwater aquatic habitats in both temperate and tropical regions (Vitt & Caldwell 2013). Amphibians typically undergo a complex lifecycle which includes a larval aquatic stage and undergo drastic changes in physiology during metamorphosis. As of early 2016 there are 7, 517 described species of amphibians (amphibiaweb.org) with new species still being discovered at a rate of >120/year over the last ten years. All amphibian species are carnivorous or omnivorous as adults, although some juvenile stages are herbivorous/detritivorous or forego feeding altogether. Thus, while amphibians represent a diverse range of taxa found in a variety of environments, they show fundamental similarities (primarily carnivorous/omnivorous, larval aquatic stage, and subject to environmental fluctuations in temperature) that could influence their associated microbial communities.

Little is known of the microbiomes of amphibians, and the majority of studies on microbial communities associated with amphibians have focused on the cutaneous (skin) microbiome (Table S1, Appendix). Even within those studies, many have utilized culture-dependent techniques to test for the presence of bacteria with anti-fungal properties or antimicrobial peptides (Culp *et al.* 2007; Brucker *et al.* 2008; Harris *et al.* 2009; Hacioglu & Tosunoglu 2014). These studies have largely been motivated to investigate factors associated with amphibian chytrid fungus, an emergent pathogen caused by *Batrachochytrium dendrobatidis* that has been linked to global declines in amphibian populations and widespread

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species' extinction (Briggs *et al.* 2010; Olson *et al.* 2013; Jani & Briggs 2014). Few studies have utilized culture-independent techniques to characterize the microbial communities found on amphibian skin (or any other part of the host) in a natural setting (Table 1, Table S1 Appendix). This lack of culture-independent studies is alarming, as it has long been recognized that the majority of bacteria cannot be readily cultured using standard techniques (Amann *et al.* 1995; Pace 1997) and only 3-7% of bacterial species identified by 16S rRNA gene analyses of cutaneous communities were likely cultivable (Walke *et al.* 2014).

Exogenous microbiomes of amphibians

The few studies that have investigated natural amphibian populations suggest that the microbial communities living on adult amphibian skin are likely to be host-species specific rather than simply being bacteria acquired from the environment. (McKenzie *et al.* 2012; Kueneman *et al.* 2014). The opposite may, however, be true for larval amphibians, whose skin communities, like those of fish, have been found to be at least partially comprised of bacterial species found in the surrounding environment (Kueneman *et al.* 2014). In a study of the skin microbiome of terrestrial red-backed salamanders (*Plethodon cinereus*), much of the bacterial diversity was derived from that found in the soil where the host lived (Loudon *et al.* 2014). That said, 90% of the OTUs identified were shared across 65 individuals, suggesting that there was some core bacterial community on the salamanders skin. The same study also showed that the composition of the amphibian skin microbiome changed during captivity, regardless of the environment (natural or artificial) in which the host was raised (Loudon *et al.* 2014). Again, this raises the question of the usefulness of microbiome studies on captive animals.

Endogenous microbiomes of amphibians

Even less is known about the gut microbiome of amphibians, with the gut community of just a single amphibian species (the leopard frog, *Lithobates pipiens*) being investigated in two studies (Kohl *et al.* 2013, 2015). The first study found that leopard frog gut bacterial communities undergo significant changes throughout metamorphosis, presumably related to physiological and environmental changes to the host. Gut communities of larval *L. pipiens* were largely comprised of bacterial species found in the water column in which they reside, while the gut bacterial communities of adults were unique and composed of species significantly different from that of the environment (Kohl *et al.* 2013). Larval amphibians from two other species (*Bufo terrestris*, *Pseudacris crucifer*) experienced an increase in the abundance of Gram-negative bacteria during metamorphosis, a shift that could have occurred as a result of depressed immune system function during metamorphosis that might allow for increased colonization of certain bacteria in the gut (Fedewa 2006). Elevated bacterial diversity in the gut of the leopard frog occurred following exposure to the pollutant polychlorinated biphenyl (PCB), which also could be a response to a weakened host immune system (Kohl *et al.* 2015). The immune system of adult amphibians fundamentally resembles that of mammals (Colombo *et al.* 2015) with the gut immune components being the largest immune system compartment (Weiner *et al.* 2011). Amphibians might therefore be expected to be excellent models for investigations on relationships between the gut microbiome and immune system function, which makes the lack of studies on amphibians even more surprising.

Diet has been shown to strongly influence gut bacterial community composition in other vertebrates (Ley *et al.* 2009; Sullam *et al.* 2012; Mikaelyan *et al.* 2015), but the impact of diet on the microbiomes of amphibians has not been addressed. Metamorphosis from larvae to adults

typically includes dietary changes, but broader developmental changes during that process likely have a greater impact than diet alone. Bacterial isolates obtained from the skin of frogs fed a carotenoid rich diet differed significantly from those of wild populations of the same host species and consisted of more bacterial species, a finding that the authors suggested might be beneficial (Antwis *et al.* 2014). Dietary and developmental changes as well as exposure to environmental contaminants certainly have the potential to influence the amphibian microbiome (both cutaneous and gut), but no broad conclusions can be drawn from such a limited amount of research. Studies have also generally been limited to the analysis of just one region of the amphibian host (i.e. either the cutaneous microbiome or the gut microbiome, but rarely both). An exception is a study by Montel Mendoza *et al*. (2012) that investigated culturable bacteria in multiple regions of an amphibian host, examining both the skin and the cloacae of captive bullfrogs (*Lithobates catesbeianus*). The cloaca (the opening for both digestive and reproductive tracts in amphibians) harbored strains of lactic acid bacteria not found on the skin (Montel Mendoza *et al.* 2012). As with many studies, however, it was limited to culture-dependent analyses of captive animals, although the finding that amphibians would have different bacterial communities in different parts of the body is not surprising given that clear differences are seen in different regions of the human microbiome (The Human Microbiome Project Consortium 2012; Cho & Blaser 2012).

Reptiles

Extant, non-avian reptiles (amphisbaenians, lizards, snakes, crocodilians, turtles and the Tuatara) are amniotic ectothermic vertebrates which occupy every continent except Antarctica, and nearly all biomes including terrestrial, freshwater and marine habitats (Vitt & Caldwell 2013). As of early 2016 there are 10, 272 described species of living reptiles making them nearly

twice as diverse as mammals (reptile-database.org) and the second most diverse clade of amniotic vertebrates behind birds (Pincheira-Donoso *et al.* 2013). Reptiles utilize a wide range of life history and reproductive strategies including sexual and asexual reproduction and in some cases have the ability to shift parity mode, a fairly plastic trait in this group. Reptiles have a variety of feeding strategies, including herbivory and omnivory, but the vast majority of species are carnivorous.

In contrast to amphibians, reptile associated microbiome studies have focused on the gastrointestinal tract and, to our knowledge, there are no published studies to date on the cutaneous microbiomes of non-avian reptiles. This is surprising, as snake fungal disease, an emergent pathogen caused by *Ophidiomyces* fungi, is rapidly spreading in the eastern United States (nine states to date since 2009) and has been identified as a potential global threat to snake populations and of high conservation concern (Rajeev *et al.* 2009; Sleeman 2013; Sutherland *et al.* 2014). Studies of the skin microbiome could provide insights into both the spread and potential susceptibility of the reptile host to this disease, in much the same way that studies of the amphibian skin microbiome may provide insights into chytrid fungus disease. Rather, studies of the microbiome of reptiles have focused on the endogenous microbial community so that, much more than amphibians, some patterns in the gut microbiome of reptiles are becoming apparent.

Endogenous microbiomes of reptiles

Culture-independent examination of the gut microbiota of two crotaline snake species by DGGE of amplified 16S rRNA gene fragments and subsequent sequencing found that while the species differed in community composition, the dominant bacterial phyla in both snakes were Bacteroidetes and Firmicutes (Hill III *et al.* 2008), the same phyla that dominate the gut microbiome of terrestrial mammals. A cross-taxonomic survey examining the presence of

members of the Bifidobacteria in various animals also used DGGE, in combination with quantitative PCR, to assess the gut microbial community of single individuals of eight lizard species and four turtle species housed in the Prague Zoo (Kopečný *et al.* 2010). Bifidobacteria comprised up to 22% of the bacteria found in the digestive tracts of the studied reptiles, and that bacterial group was also found to be abundant in the digestive tracts of wasps, cockroaches, and bumblebees (Kopečný *et al.* 2010). While the insects sampled were collected from wild populations, insects are a major food item for captive reptiles so these findings may be correlative (i.e. the gut microbiome of prey could contribute to the gut microbiome of a predator). However, Bifidobacteria are also often the dominant bacteria in the gut of human infants (Sela *et al.* 2008; Yatsunenko *et al.* 2012) and their presence in captive reptiles could reflect a limitation in diet breadth as well as specific dietary composition.

DGGE has substantial limitations compared to next-generation sequencing and provides only a cursory overview of the diversity of a microbial community. The first use of NGS to examine the gut microbiome of reptiles investigated how the gut microbiome of Burmese pythons (*Python bivittatus*) changed during the digestion of prey items (Costello *et al.* 2010). As is the case for almost all of these studies, the animals were housed in captivity, although to some extent the study confirmed the work of Hill III *et al.* (2008), and found that the python gut microbiome was dominated by members of the bacterial phyla Bacteroidetes and Firmicutes. However, the relative abundances of these phyla and overall bacterial species diversity changed significantly during digestion, with an overall increase in abundance and diversity of Firmicutes during the digestive process (Costello *et al.* 2010). The increase in diversity could not be attributed to bacteria found in or on the prey item, so the observed changes in bacterial community composition likely represent shifts in bacterial populations indigenous to the host

rather than an accumulation of those associated with the meal (Costello *et al.* 2010). At a finer scale, the study also investigated bacterial community composition in different regions of the GI tract, sampling both the small and large intestines which were found to have similar phylum level bacterial composition (Costello *et al.* 2010). Members of the Bacteroidetes only dominated the large intestine during fasting periods, with Firmicutes (largely members of *Clostridium, Lactobacillus* and the Peptostreptococcaceae) gradually outnumbering the Bacteroidetes during periods of active digestion. This pattern was also observed in the small intestine, with Bacteroidetes dominating in fasting individuals, although the authors lacked sufficient sampling in the small intestine to test this statistically (Costello *et al.* 2010).

A more detailed examination of the differences in microbiome composition in different regions of the gut was performed on a wild crotaline snake, the cottonmouth (*Agkistrodon piscivorus*; Colston *et al.* 2015). Next generation sequencing was used to examine multiple samples taken from the small intestine, large intestine, and cloaca. As found by Costello *et al.* (2010), members of the phylum Bacteroidetes were the dominant bacteria of the large intestine; however, the composition of the small intestine differed from previous findings in that the Firmicutes were not the dominant bacterial phylum present (Colston *et al.* 2015). Rather, bacteria belonging to the phylum Proteobacteria dominated samples taken from the small intestine and cloaca (the single opening for both excretory and reproductive organs in reptiles). The increased prevalence of Proteobacteria suggests a gut microbiome more similar to that of birds (Hird *et al.* 2014), and also suggests that, again, the enteric microbiomes of wild individuals may be substantially different from those of animals raised in captivity (Colston *et al.* 2015). This question is intriguing both from an evolutionary standpoint and from that of experimental design. The earlier, limited studies that suggest that reptiles share gut microbiota similar to terrestrial

mammals should probably be re-investigated using samples collected from wild rather than captive hosts, and across a broader taxonomic range.

Dietary shifts are known to drive speciation in animals and shifts from carnivory or omnivory to herbivory lead to shifts in the gut microbiome of mammals (Ley *et al.* 2008). While the majority of extant reptiles are carnivorous, a small number (roughly 2% of known species) are herbivorous (Stevens & Hume 2004). Examination of the feces of herbivorous marine iguanas (*Amblyrynchus cristatus*) and land iguanas (*Conolophus subscristatus* and *C. pallidus*) of the Galapagos Islands suggested gut microbiota dominated by members of the Firmicutes (Hong *et al.* 2011). Fecal microbial composition was dependent on host species and land and marine iguanas, as well as terrestrial tortoises occurring on the same islands, each harbored specific bacterial communities. The land iguanas also showed some similarities in fecal microbial composition to terrestrial iguanas from a different species and locality (Hong *et al.* 2011). These results suggest potential similarities between the gut bacterial communities of herbivorous reptiles and those of herbivorous mammals. Fecal microbial communities of the iguanas also varied according to geographic location of the host, and while the primary differences in bacterial community structure were related to host species and ecotype (marine or terrestrial), within each species or ecotype, bacterial communities were structured by geographic location, with more proximal hosts having a more similar fecal microbial community (Lankau *et al.* 2012). This suggests either localized environmental impacts on the gut microbiome or the potential exchange of gut populations between hosts that are spatially closer. The latter could indicate some degree of microbiome heredity if spatially closer individuals are genetically related, or the exchange of gut bacteria through mechanisms such as shared feeding or coprophagy. While either concept seems plausible, similarity in gut microbiota between individuals that are

geographically close together has not always been found for other reptiles. For example, while herbivorous gopher tortoises (*Gopherus polyphemus*) appear to have similar gut microbiota to other herbivorous reptiles, their microbial communities showed no relationship to geographic locality of the host or to local plant variation (Gaillard 2014).

Gopher tortoises may represent an interesting variation in reptile microbiome composition, as while members of the Firmicutes and Bacteroidetes dominate their fecal microbiota, these taxa have been found in near equal proportions (Yuan *et al.* 2015). This is unusual as other herbivorous reptiles tend to have fecal communities that are overwhelmingly dominated by Firmicutes (Hong *et al.* 2011). However, turtles and tortoises have the highest proportion of herbivorous species among major reptile lineages (Vitt & Caldwell 2013), so this trend of a more taxonomically balanced gut microbiome may be more widespread in herbivorous "grazing" reptile species than is typically assumed. Bacterial species richness was found to be higher in samples from adult gopher tortoises compared to juveniles (Yuan *et al.* 2015), a pattern that has also been noted in studies of the human gut microbiome (Koenig *et al.* 2011; Yatsunenko *et al.* 2012). While gut bacterial community composition in gopher tortoises was not strongly related to geographic distance between hosts, a weak relationship existed between gut microbiomes and conservation management practices (prescribed fire treatments) of the environment (Yuan *et al.* 2015). The lack of broader geographic patterns in gopher tortoise gut bacterial community structure may be a function of the ecology of this species, which can traverse great distances and often have large home ranges. The same study also found a weak association between microbiome structure and kinship, with parent-offspring and full siblings showing similar microbiome structure. These relationships could have arisen from direct parental

transmission during egg development, sibling association in the nest, or coprophagy biased towards close kin (Yuan *et al.* 2015).

Whether comparing genetically related individuals or not, little work has been done on understanding how reptiles acquire their associated microbial community. For mammals, acquisition of the gut microbiome begins during the birth process, and the infant microbiome develops further following nursing and other maternal contact. Broader environmental acquisition continues to occur so that infants begin to develop a microbiome that resembles that of cohabiting individuals, not just that of the mother (Koenig *et al.* 2011). The gut microbiome of juvenile reptiles likely develops from environmental exposure, for example, hatchling iguanas eat soil as they exit the nest and also acquire kin-associated microbes through coprophagy (Troyer 1984). Coprophagy also occurs within several turtle and tortoise species, although whether this is the primary mode of acquisition of gut microbiota remains untested (Yuan *et al.* 2015). This generalization remains underexplored in other reptile lineages where other mechanisms for microbiome acquisition may be present. For example, coprophagy is unlikely to be present in strictly carnivorous species, and the acquisition of bacterial populations from prey items may be more important.

Of the 25 species of extant crocodilians (Vitt & Caldwell 2013) microbiome analyses have only been performed in the American alligator (*Alligator mississippiensis*; Keenan *et al.* 2013; Keenan & Elsey 2015). However, the microbiome of this species has been quite well studied, with investigation of the bacterial community along the entire GI tract from mouth to cloaca in both wild and captive individuals, as well as during winter and spring months which represent periods of fasting or active feeding, respectively (Keenan *et al.* 2013). The oral, upper GI tract and lower GI tract harbored distinct bacterial communities, with the mouth containing

the richest bacterial community, presumably because of its exposure to the aquatic environment (Keenan *et al.* 2013). The microbiome of the oral cavity and upper GI tract were dominated by members of the Proteobacteria, while the lower GI tract was more variable (Keenan *et al.* 2013). In feeding alligators, the lower GI tract microbiome was dominated by members of the Firmicutes and Fusobacteria in both wild and captive individuals, although a dramatic increase of Firmicutes in wild individuals once feeding began in spring was not observed in captive individuals, whose proportion of Firmicutes remained relatively constant. The unexpected prevalence of Fusobacteria rather than Proteobacteria or Bacteroidetes in the gut of wild alligators may reflect the hosts ecology, as wild alligators frequently feed on carrion and Fusobacteria have previously been found to comprise the majority of the endogenous microbiome in vultures, another carrion feeder (Keenan *et al.* 2013; Roggenbuck *et al.* 2014).

During periods of fasting, the lower GI tracts of both wild and captive alligators were dominated by members of the Proteobacteria and Bacteroidetes (Keenan *et al.* 2013). The much reduced shifts in microbiome structure of captive alligators vs. wild individuals during fasting or feeding months again calls into question the usefulness of microbiome data acquired from captive animals. Furthermore, when fecal samples were considered alone, they were overwhelmingly dominated by Bacteroidetes, although Bacteroidetes represented less than 10% of the composite microbiome when all other GI regions were included. This could lead to a false impression of the "gut microbiome" if only fecal samples are considered, even though characterizing the endogenous microbiome through fecal sampling is a common practice with humans and other mammals (Ley *et al.* 2008; Arumugam *et al.* 2011a; Keenan *et al.* 2013).

Birds

Avian reptiles (birds) are the most diverse group of amniotic vertebrates with 10,425 described species and more than 20,000 subspecies varieties (avibase.org). Birds are endothermic, feathered amniotes with a global distribution and many species undergo lengthy seasonal migrations across great distances. While birds feed on a variety of diets, dietary preferences are often related to body size, with smaller species (e.g. hummingbirds) tending to be herbivorous and larger species (e.g. eagles) being carnivorous, with the exception of flightless birds (Stevens & Hume 1998). Birds exhibit parental care to a greater degree than other vertebrates with the exception of mammals, and these factors could be presumed to have important relationships to their associated microbial communities.

Compared to other non-mammalian vertebrates, our understanding of the enteric microbiome of birds is greater, although the majority of avian microbiome studies have focused on economically important species such as chicken and turkey. The impacts of diet, probiotic treatment, kinship and captive rearing conditions on the endogenous microbiomes of poultry has been reviewed elsewhere (Brisbin *et al.* 2008; Cisek & Binek 2014) and given the highly artificial conditions in which poultry are reared are likely of little benefit in understanding the evolution and ecology of avian microbiomes in general. The wide variety of diets and life history strategies employed by birds make them of particular interest to microbiome research, but although recent studies have capitalized on the NGS revolution (Benskin *et al.* 2010; Hird *et al.* 2014; Waite & Taylor 2014), as with other taxa, most published studies of avian endogenous microbiomes have used culture-dependent approaches or limited to Sanger sequencing of 16S rRNA gene clone libraries (Figure 1, Table 1, Table S1 Appendix). Those studies do reveal the dominance of bacteria belonging to the Bacteroidetes and Proteobacteria in the avian GI tract,

and while members of the Firmicutes are typically present in any avian sample, samples from

captive poultry contain higher proportions of Firmicutes than do those acquired from wild birds

(Waite & Taylor 2015). Vultures may be an exception, and have high proportions of Clostridia (a

class of Firmicutes) in their hindgut, presumably an adaption to feeding on carrion (Roggenbuck

et al. 2014). Ordination analyses of the earlier avian microbiome sequence data has revealed that

gastrointestinal microbial communities group by sampling region (crop, ceca, cloaca, fecal;

Waite & Taylor 2014), a result shared with reptiles (Costello *et al.* 2010; Colston *et al.* 2015) and

mammals (Ley *et al.* 2008). The same data also separated into gut communities sampled from

captive vs. wild individuals (reinforcing the idea that microbiome studies of captive vertebrates

may be of limited use in extrapolating to wild populations) and into microbiome assemblages

from carnivorous vs. omnivorous bird species (Waite & Taylor 2014).

Endogenous microbiomes of birds

One of the first culture-independent studies to investigate the gut microbiome of wild

birds, examined seasonal changes in the gut microbial community of capercaillie (*Tetrao*

urogallus) examining both wild and captive individuals (Wienemann *et al.* 2011). Wild birds

showed differences in bacterial community composition in summer vs winter months, likely

because of drastic dietary shifts, which the captive individuals did not experience (Wienemann *et*

al. 2011). Differences in the proportions of specific taxa between wild and captive individuals

were also observed, with members of the Clostridiales, Synergistetes, and Actinobacteria being

abundant in wild birds, and significantly reduced in captive individuals, whose gut microbiome

was dominated by members of the Gammaproteobacteria. The finding that Actinobacteria are

abundant in the GI tract of wild birds was further supported by a study on the cloacal

microbiomes of barn swallows (*Hirundo rustica*), where Proteobacteria, Firmicutes and

Actinobacteria were the dominant bacterial phyla (Kreisinger *et al.* 2015). Once again these findings highlight the necessity to use caution when drawing inferences from microbiome studies of captive animals, and also raise the question as to whether conservation planners should incorporate microbiome analyses into management plans, particularly for endangered species that may be raised in captivity for re-introduction into the wild.

The only study to characterize the gut microbiome of volant seabirds, a highly divergent group of birds that are unique in their ability to produce stomach oils through partial digestion of prey which aids in trans-oceanic dispersal, found GI bacterial communities to be dependent on host species (and thus the hosts' ability to produce stomach oils; Dewar *et al.* 2014). Firmicutes and Bacteroidetes dominated the bacterial community across all host species, although communities also contained high proportions of Proteobacteria (5-30%). The significant differences found between gut microbial communities of oil-producing and non-oil producing seabirds was likely not just a reflection of host species or digestive physiology, but rather average retention time of ingesta (Dewar *et al.* 2014). Retention time of food within the GI tract could have implications for the endogenous microbiome of many vertebrate taxa, and is an intriguing concept that has yet to be explored in other taxa which feed infrequently. A combination of qPCR (to measure overall bacterial abundance) and NGS analyses have been used to examine the gut microbiota of penguins (Dewar *et al.* 2013) which also have the ability to store food for long periods. The four penguin species investigated (king, *Aptenodytes patagonicus*; Gentoo, *Pygoscelis papua*; macaroni, *Eudyptes chrysolophus*; and little penguin, *Eudyptula minor*) shared the same dominant bacterial phyla (Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria) but the proportion of these bacterial phyla varied greatly by species (Dewar *et al.* 2013). Each penguin species harbored a distinct GI microbiome with

overlap between host species ranging from as little as 10% (king and all other species) to 50% (between Gentoo and macaroni penguins). As well as harboring the most distinct gut microbial community, king penguins also showed the lowest diversity in their microbiomes. The distinctness of the king penguin microbiome from that of the other penguin species examined could be a reflection of host phylogenetic distance or trophic position, as higher predators often have more prey-associated microbiota and lower overall diversity (Dewar *et al.* 2013, Nelson 2006).

The facial microbiomes of two species of wild vulture (*Coragyps atratus* and *Cathartes aura*) showed greater bacterial diversity than gut microbiomes of the same species, although both facial samples and hindgut samples were dominated by Clostridia and Fusobacteria (Roggenbuck *et al.* 2014). This finding was most attributed to the ecology of vultures, which primarily feed on carrion and often open carcasses from anal orifices (Roggenbuck *et al.* 2014). Facial and hindgut samples were also collected from captive individuals of the same vulture species, as well as from several additional predatory bird species. Although all predatory birds were fed similar diets in captivity, bacterial communities were found to be host-species specific, with only captive vultures having the abundance of Clostridia and Fusobacteria that characterized the microbiomes of wild vultures. This finding is in contrast to other studies on captive vs. wild bird microbiomes (Kreisinger *et al.* 2015), or the overwhelming support for differences between the microbiomes of wild and captive individuals for other vertebrates. It suggests that for certain host lineages, the phylogenetic signal in their associated microbiomes may be greater than an environmental or dietary signal that is subject to change during captivity.

Unlike other vertebrate taxa (mammals, fish and reptiles) where host genetics or phylogeny have shown a clear influence on gut microbial community structure (Ley *et al.* 2008;

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Arumugam *et al.* 2011b; Wong & Rawls 2012), the evidence for this association is generally

lacking in birds (Waite & Taylor 2015). Avian host-specific gut bacterial communities appear to

be more of a reflection of diet and geography (Hird *et al.* 2014; Waite & Taylor 2014) rather

than phylogeny, which may reflect differences in reproductive physiology and/or offspring

rearing in birds compared to other vertebrates, although this idea remains unexplored. It is

assumed that birds acquire their gut microbiota from the nest environment or from food

consumed after hatching, although few studies have tested this (Kohl 2012). In cowbirds

(*Molothrus ater*), a brood parasite which relies on other species to hatch and raise their young,

gut microbial community composition is not related to host species or ecology, but rather to

geographic location, lending support to the theory that birds primarily acquire their gut

microbiota from their immediate surroundings post-hatching (Hird *et al.* 2014). However, many

hatchling birds feed on regurgitated food items from their parents, which potentially provides a

mechanism for vertical transmission of gut microbiomes across generations (van Dongen *et al.*

2013). Gastrointestinal microbiota can also be transferred between individual birds during sexual

copulation, providing another avenue for the acquisition of both beneficial bacteria as well as

potential pathogens (White *et al.* 2010). This was shown to be the case in barn swallows, where

nesting pairs had significantly more similar microbiomes within pairs than between non-breeding

individuals (Kreisinger *et al.* 2015). The potential exchange of components of the endogenous

microbiome during reproduction has not been explored in other terrestrial vertebrates (reptiles,

amphibians) that have cloacae that are used for both reproductive and excretory functions.

ECOLOGICAL AND PHYSIOLOGICAL FACTORS INFLUENCING THE VERTEBRATE

MICROBIOME

Diet

Studies on mammals suggest that host diet can have a profound effect on the gut microbiome, with recognized carnivore and herbivore mammalian gut types and an increase in microbiome diversity from carnivores through omnivores through herbivores (Ley *et al.* 2008; Muegge *et al.* 2011). These findings are also supported by surveys of human populations, and differences in the microbiome associated with a typical high fat, high protein "Western" diet compared to those of more agrarian cultures have been reported (Yatsunenko *et al.* 2012). Indeed, the development of agricultural practices and associated dietary change may be one of the most important drivers in the recent evolution of the human-microbiome symbiosis (Walter & Ley 2011). Based on human studies, it is clear that differences in dietary preferences and practices within a species can result in the development of substantially different gut microbiomes. How this translates to comparisons between different species of vertebrates has rarely been addressed. Do different species that share the same general dietary preferences also share some components of their microbiome?

At least at a very broad level this seems to be the case, and the GI tract of most vertebrates is dominated by members of the Firmicutes, Bacteroidetes and Proteobacteria regardless of whether the host is herbivorous or omnivorous, although the proportion of each of these groups varies substantially (Ley *et al.* 2009; Hird *et al.* 2014; Colston *et al.* 2015). As well as the general patterns seen for mammals between carnivorous and herbivorous diets (Ley *et al.* 2008), phylogenetically distant mammals which have converged on highly specialized diets (e.g. ants) have been found to have highly similar gut microbiomes (Delsuc *et al.* 2014). Whether or

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not similar convergence occurs at a broader phylogenetic range of vertebrates has not been addressed with data from wild hosts. Human studies suggest that the members of the Bacteroidetes that are present in the GI tract are often responsible for carbohydrate fermentation, degrading plant-derived material and potentially producing short chain fatty acids that can be absorbed by the host and even contribute to its nutrition (Walter & Ley 2011). However, this is not so clear in birds, and two herbivorous foregut fermenting species (the South American hoatzin, *Opisthocomus hoazin*, and New Zealand's kakapo (a presumed foregut fermenter), *Strigops habroptilus*) have been found to have significantly different endogenous microbiomes despite similar dietary strategies (Godoy-Vitorino *et al.* 2012; Waite *et al.* 2012). In contrast, members of the Firmicutes may be more responsible for protein degradation, and the gut bacterial communities of terrestrial carnivorous mammals contain greater proportions of Firmicutes than those of terrestrial herbivorous mammals (Nelson *et al.* 2013). However, both of these bacterial phyla contain many different subgroups of bacteria, including species with a variety of properties so that these distinctions are not absolute (e.g. there are members of the Firmicutes that ferment plant polysaccharides and whose numbers increase when on an herbivorous diet; David *et al.* 2014).

There has been little interest in examining how changes in diet can influence the gut microbiome in vertebrates other than humans, or how changes to one part of the microbiome might influence that of another anatomical region. Cutaneous and mucosal microbiomes play an important role in disease resistance of the host (The Human Microbiome Consortium 2012) and changes in the gut microbiome of humans can be correlated with changes in the microbiome of other body regions (Cho & Blaser 2012; Clemente *et al.* 2012). It is unclear as to whether similar shifts occur in the microbiomes of other organisms, or how linked these communities are.

Humpback whales (*Megaptera novaeangliae*) show significant shifts in their skin microbiomes during long periods of fasting vs active feeding, possibly reflecting stress or reduced health during fasting periods (Apprill *et al.* 2014). Whether such changes occur in non-mammalian vertebrates that feed intermittently (e.g. many reptile species) has yet to be explored. The gut microbiome of Burmese pythons changes during periods of fasting and feeding (Costello *et al.* 2010) suggesting that changes to other compartments of the host microbiome are possible. An active area of research in human microbiome studies are the links between the gut microbiome and the endocrine system, and ultimately host behavior (Lyte 2013; Foster & McVey Neufeld 2013). Given the importance of hormonal cues in the behavior of many vertebrate taxa, it's certainly possible that diet-induced changes in the gut microbiome could have far-reaching impacts for many non-mammalian species.

Temporal patterns in the microbiome of animals that feed intermittently is another area that is poorly understood. Many vertebrates undergo cycles of feeding and fasting, a feeding pattern that is common in reptiles but also seen in amphibians and fish. Studies on mammals have shown changes in endogenous microbial community structure following fasting (Morishita & Miyaki 1979; Sonoyama *et al.* 2009) and the same has been suggested for fish (Xia *et al.* 2014) and reptiles (Colston *et al.*; Costello *et al.* 2010; Keenan *et al.* 2013). Extended periods of fasting are likely to lead to substantial reductions in nutrient availability to the endogenous microbiome, potentially leading to changes in both overall diversity and phylogenetic composition. In a comparison across different classes of vertebrates, Kohl *et al.* (2014) showed that fasting increased diversity in the colon microbiome of fish (tilapia, *Oreochromis niloticus*) and amphibia (southern toads, *Anaxyrus terrestris*), but decreased diversity in the colon microbiome of birds (quail, *Coturnix coturnix*), and had no effect on a reptile (leopard geckos,

Anaxyrus terrestris). While that study suggested some common responses of the vertebrate gut microbial community to food availability (decreases in the relative abundance of genera such as *Ruminococcus* and *Coprobacillus*), comparisons across different vertebrate classes are of limited value without knowledge of the variation in the response between species within each class. Each class of vertebrates show substantial variation in species that feed often or occasionally, suggesting that class-scale comparisons are of only minimal value. This is compounded by the effects of dietary composition; and if diet influences the composition of the vertebrate microbiome then lab-reared animals (generally on a defined and restricted diet) are not likely to be at all representative of those in a natural setting. That said, the finding that geckos, organisms that have a more opportunistic diet, showed only minimal changes in their gut microbial community compared to other vertebrates (Kohl *et al*. 2014) does suggest possible evolutionary adaptation of the endogenous microbiome to host feeding strategy. Regardless, care must be taken when sampling wild individuals to note (if possible) their feeding state as this could profoundly impact their gut microbiome composition.

Life History and Ontogeny

Vertebrates go through a variety of physiological transformations throughout ontogeny which influence microbiome composition (Stevens & Hume 1998). These changes may be gradual, as in the case of placental mammals, or extreme as in amphibian metamorphosis. Little attention has been given to how non-mammalian vertebrates acquire their microbiome, or how ontogenetic shifts affect its composition. Even in adults, dietary shifts because of migration or other life history strategy (i.e. sneaker male toads, fishes) are accompanied by a suite of physiological and hormonal changes (Plaistow *et al.* 2004) which could influence microbiome structure. The extent to which the microbiome could influence these changes and vice versa has

not been investigated outside of mammals. In humans it is generally thought that it takes long term dietary changes to influence the core gut microbiome (Walter & Ley 2011) although significant shifts have been observed in as little as five days (David *et al.* 2014). As humans age, decreased immunity and other physiological changes may be related to shifts in the microbiome (Heintz & Mair 2014). There are non-mammalian vertebrates with lifespans that equal or significantly exceed that of humans (e.g. tortoises >100 years). It would be interesting to investigate the microbiome composition across a variety of age classes in other long-lived species.

Ecological speciation is often accompanied by shifts in life history traits (Rundle & Nosil 2005; Schluter 2009), and could be accompanied by or even driven by shifts in the microbiome (Brucker & Bordenstein 2012). Yet few studies have investigated shifts in the vertebrate microbiome in the context of ecological speciation. In one such study, the kidney-associated bacterial communities of sympatric species pairs (dwarf and normal) of lake whitefish (*Coregonus clupeaformis*) were investigated across five lakes, to test whether ecologically divergent forms differed in their microbiomes (Sevellec *et al.* 2014). While an effect of lake/locality was significant, the differences in bacterial composition between lakes were not the same for the two ecological species, suggesting form-level variation (Sevellec *et al.* 2014). Speciation in poeciliid fishes provides another example of shifts in physiological function and ecology, as species have diverged along a continuum of freshwater to water containing high levels of hydrogen sulfide (toxic to most other fish species) as well as both freshwater and toxic caves (Tobler *et al.* 2008). This has led to a multitude of shifts in life history and physiology including fecundity, offspring size, feeding performance and behavioral adaptations (Riesch *et al.* 2010). It would be interesting to investigate the microbiome composition of the different host

ecotypes in this system and how it relates to host fitness, as well as changes to the microbiome structure along this natural toxicity gradient.

Reptiles and amphibians regularly experience ecdysis or sloughing of their skin throughout their lifetime (Vitt & Caldwell 2013). Although the amphibian skin microbiome has been extensively investigated for its role in disease resistance, only cursory attention has been paid to the turnover in skin-associated microbiota during sloughing. Culturable bacteria present on the skin of marine toads (*Rhinella marina*) changed significantly after sloughing; reductions in culturable numbers of up to 100% occurred in some individuals post-sloughing (Meyer *et al.* 2012). That would imply an almost cleansing of the skin microbiome during sloughing events, which has important implications not only for natural biological resistance against pathogens, but also for the effective administration of probiotics to combat emergent disease.

Many mammals, birds, amphibians and reptiles are known to actively suppress their metabolism during winter or other periods of inactivity in order to gain energetic benefits during non-feeding periods (Lyman *et al.* 1982; Vitt & Caldwell 2013; Ruf & Geiser 2015). While this reduction in metabolism is less widespread in fish, suppression of metabolism during winter inactivity has been documented (Campbell *et al.* 2008). A reduction in metabolism could have substantial influence on the composition of the microbial community anywhere in the host, but this has yet to be explored in detail. The effects of short term fasting on the gut microbiome have been investigated across several vertebrate classes (Kohl *et al.* 2014), but studies investigating temporal changes to the microbiome throughout hibernation or torpor have been limited to mammals. Seasonal reductions in the microbial diversity of the gut lumen and gut mucus associated community have been documented in ground squirrels (*Ictidomys tridecemlineatus*), which was coupled with a decrease in the proportions of Firmicutes and increase in Bacteroidetes

and Verrucomicrobia (Carey *et al.* 2013; Dill-McFarland *et al.* 2014). Despite those seasonal shifts, a core microbiome comprised of OTUs present in all seasons was identified in the gut mucosa, the region of the GI tract that is more closely associated with the host's epithelial cells and has a stronger influence on the hosts immune response (Dill-McFarland *et al.* 2014). Although changes to Toll-like receptors (TLRs) in response to shifts in the gut microbiome were not explicitly tested, increases in TLR5 receptors during hibernation suggests that shifts in the microbiome may contribute to a decreased inflammatory response during hibernation (Dill-McFarland *et al.* 2014). The role and persistence of a core gut microbiome throughout hibernation is an avenue of research that has yet to be thoroughly explored outside of mammals.

The skin microbiome plays an important role in host defense and changes to the bacterial composition on the skin of hibernating bats have been investigated (Hoyt *et al.* 2015). Cultured bacteria from four species of bats in hibernacula were shown to have inhibitory effects on the fungal pathogen *Pseudogymnoascus destructans*, the causative agent of white-nosed syndrome, a disease which is causing widespread population extinctions in hibernating bats (Hoyt *et al.* 2015; Frick *et al.* 2015). Again, outside of mammals, we are not aware of studies investigating changes to the skin microbiome of other hibernating vertebrates or their implications for host disease resistance during prolonged periods of torpor.

CONCLUSIONS

We have presented a survey of the literature on non-mammalian vertebrate microbiomes. Much of this discussion has focused on the evolution of the gut microbiome, and the evidence of microbial interactions with ancient host lineages leading to convergent microbial assemblages across a wide range of taxa (Rawls *et al.* 2004; Ley *et al.* 2008, 2009; Costello *et al.* 2010). However, these studies drew largely from captive animals in zoos or laboratories, and we question whether these relationships are as clearly partitioned in the natural world. The hypothesis that animals harbor a "core" microbiome that is reflective of phylogeny or ecology is intriguing nonetheless and there is mounting evidence that this is the case in natural populations, although significant variations exist across vertebrate classes. Whether the core microbiome is more reflective of ecology or phylogenetic history is likely linked to how an organism acquires its microbiome, via vertical transmission as in mammals or largely from the environment as appears to be the case in fishes. However, this area is largely understudied and unknown. Through NGS technologies it is now becoming relatively inexpensive to characterize host associated microbiomes. We no longer need to rely on culture-based methods to characterize the microbiome. Researchers need to work together to develop standardized methods that aim to reduce taxonomic bias introduced from variation in sample type and collection method, as well as in DNA extraction and sequencing protocols, in order to accumulate datasets that are complementary in order to facilitate reliable meta-analyses of the vertebrate microbiome in natural populations.

Box 1 – Variation in Non-Mammalian Vertebrate Digestive Tracts and the "Gut" Microbiome

Variation in the vertebrate digestive tract and its relevance to physiological function has been reviewed elsewhere (Stevens & Hume 1998), but given that such variation may impact the structure of the gut microbial community, it is important to highlight key differences both between and within major vertebrate classes and how "gut" microbiome sampling varies. A number of studies have investigated the variation in gut microbiome along different regions of the GIT (Waite *et al.* 2012; Kohl *et al.* 2013; Colston *et al.* 2015; Lowrey *et al.* 2015) and although representatives of all major classes of vertebrates have now been investigated, these studies have not investigated the breadth of GIT variation found within these taxa (Figure 1). The variation in pH, particle retention time, and nutrient absorptive function of each region along the GIT will influence the microbiota that can survive and inhabit that environment. As digesta passes through the GIT there is an expected turnover in microbial species and abundance, and the final excrement of feces will have an expected environmental influence on the fecal microbiome. *Fish* – Nearly one half of all described vertebrate species are classified as fish. Fishes may be carnivorous, omnivorous, detritivorous, herbivorous and may vary their diet seasonally or through ontogeny. As such fishes have a wide range of digestive physiologies but generally, fish have a short esophagus that leads to a straight, U or Y shaped stomach (if present) that is lined with gastric mucosa. The midgut or intestine of fish is either short and straight or long with many loops which may form a lumen encapsulated spiral valve that may store food and delay digestion. The hindgut of fish is generally short. Herbivorous fish may have specialized gizzard like stomachs and/or pharyngeal teeth present to assist in the grinding of food. Most studies of the fish "gut" microbiome characterize the hindgut or whole intestinal tract, but some studies

include or limit the microbiome to feces (Table S1 Appendix). The variation in the region of the

16S rRNA gene sequenced has been substantial, with most studies utilizing the V1-V3 region,

but with more recent work emphasizing the V4-V5 region. Within fish gut microbiome studies

there is also variation as to whether intestinal contents, mucosa or tissue are used to characterize

gut microbiota. Each of these sampling methods would yield expectedly different results.

Amphibians – Most amphibians begin life as free-living aquatic larvae that may be carnivorous,

omnivorous or herbivorous. Larval amphibians generally lack a stomach but rather the mouth

immediately leads to a long looped, mucus-lined intestine with low pH and no distinct regions.

The GIT of amphibians undergoes restructuring during metamorphosis and adult amphibians

have a mucosa-lined stomach, shortened intestine, and defined hindgut. The few studies on the

amphibian gut microbiome have used cloacal swabs, swabs of the different gut regions, gut

tissue, whole GIT and feces (Table S1 Appendix). There is less variation in the region of the 16S

rRNA gene sequenced for amphibian gut microbiota, with studies typically utilizing the V4

region.

Reptiles – Most species of reptiles are either carnivorous or omnivorous, but a few species are

herbivorous. Most reptiles, like birds and mammals, have salivary glands which aid in the

deglutition of food as it travels the esophagus from the mouth. Reptile stomachs are tubular, and

lack a separate pylorus with the exception of crocodilians. The mucosal surface of the stomach is

divided into gastric, pyloric and occasionally cardiac regions. The midgut of carnivorous reptiles

tends to be longer than that of herbivores, with the opposite being true of the hindgut.

Herbivorous reptiles usually have a cecum and proximal colon which are defined by mucosal

folds. Reptile gut microbiomes are often characterized with fecal samples that have been exposed

to the environment, although section GIT tissue, swabs and cloacal swabs have been employed

(Table S1 Appendix). The portion of the 16S rRNA gene sequenced has typically been the V1-V4 region, with more recent studies focused on the V4 region.

Birds – Birds may be carnivorous, omnivorous or herbivorous. Functions typically carried out in the stomachs of other vertebrates, such as food storage, acid secretion and trituration are divided amongst the crop, proventriculus and gizzard in birds. The relative sizes and mucosal properties of these organs vary with diet, with herbivores typically having larger crops and muscular gizzards. The midgut of most birds is short, and the hindgut consists of a short straight colon and typically paired ceca. Within herbivorous bird species the site of microbial fermentation is known to vary substantially and may be the crop (rare), midgut, ceca or colon. Typically the site of fermentation is enlarged relative to other organs (e.g. the emu has a relatively short ceca and colon but a long midgut). The bird gut microbiome has overwhelmingly been characterized via fecal samples with the occasional use of cloacal swabs or intestinal tissue (Table S1 Appendix). While the microbiome along the GIT of domestic poultry has been investigated thoroughly, only a few studies have longitudinally sampled the GIT of other species. The variation in the region of the 16S rRNA gene sequenced has been substantial, with most studies utilizing the V3-V4 region.

Box 2 – Major Bacteria of the Vertebrate Gastrointestinal Tract

The microbiome of the vertebrate GIT is likely dominated by bacteria which aid in nutrient absorption and maintaining homeostasis. The various regions of the GIT are inhabited by a wide range of bacteria, many of which are poorly known and not culturable using standard microbiological techniques. Here we summarize the diversity and function of the major bacterial phyla common to the vertebrate GIT. Functional information is largely derived from human microbiome studies, with an excellent overview of that topic and the role of bacteria in host health provided by the Human Microbiome Consortium (2012).

Actinobacteria –Actinobacteria (formerly the "High GC Gram-positive bacteria") are typically thought of as soil bacteria but are found in most environments, including associated with animals. All members of the phylum are heterotrophs, but it includes both aerobic and anaerobic species. The phylum includes some pathogenic genera (*Corynebacterium*, *Mycobacterium*, *Propionibacterium*), and is typically a minor (<5%) component of the gut microbiome, but is much more prevalent on the skin where it can account for 50% of the human skin microbial community. The phylum has been detected in the guts of fishes and birds, as well as on the mucosa and skin of fishes. The majority of gut-inhabiting Actinobacteria are species of *Bifidobacterium*, which have been shown to aid in maintaining host homeostasis, inhibition of Gram-negative pathogens and lactic acid fermentation. In humans, *Bifidobacterium* are dominant members of the gut microbiome during infancy where they likely help metabolize milk sugars.

Bacteroidetes – Members of the Bacteroidetes are heterotrophic bacteria which carry out a range of metabolisms ranging from aerobic respiration to fermentation. The phylum was formerly named the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, which represent genera in its three dominant classes (Cytophagia, Flavobacteriia, Bacteroidia). Bacteroidetes are one of the

most abundant bacterial phyla found in the vertebrate GIT, with the genus *Bacteroides* typically being the most common. These organisms are strictly anaerobic, and have the ability to degrade complex molecules (polysaccharides, proteins) in the intestine, making them important for both herbivorous and carnivorous diets. Increased presence of *Bacteroides* has been linked to obesity in mammals, potentially from their ability to release extra energy from otherwise indigestible food (Turnbaugh *et al.* 2006), and differences between a microbiome that is predominantly *Bacteroides* with one that is predominantly *Prevotella*, a related genus, may reflect differences between a protein/fat-based and carbohydrate-based diet (Wu *et al.* 2011). Bacteroidetes also likely aid in the development of host mucosal and systemic immunity. While not commonly seen in the GIT, species of both *Flavobacterium* and *Cytophaga* are known pathogens of fishes, typically causing diseases of the skin or gills.

Firmicutes – The Firmicutes (or "Low GC Gram-positive bacteria") are typically the most abundant bacterial phylum present in the vertebrate GIT, particularly in herbivores, and are also one of the dominant phyla found on skin. Firmicutes in the GIT are typically members of the class Clostridia, obligate anaerobes utilizing fermentation as their sole metabolism, and important in the breakdown of carbohydrates and nutrient absorption. While some GIT-associated species can become pathogenic in humans following gut microbiome disturbance (e.g. *Clostridium difficile* after antibiotic use), most are commensal and have been found to be important in the maintenance of gut homeostasis and the development of immunity (Lopetuso *et al.* 2013). Other Firmicutes include the lactic acid bacteria, which while found in the GIT are typically more common on the skin. This clade (the Lactobacillales) is also the most prevalent group of bacteria in the human vaginal tract, where it is thought to play an important role in

pathogen reduction through the production of lactic acid. Interestingly, lactic acid bacteria have also been detected in the cloaca of amphibians.

Fusobacteria – Fusobacteria are one of the less abundant phyla in the typical vertebrate GIT but can account for approximately 5% of bacteria in the human oral cavity (Cho & Blaser 2012). The normal role for these organisms in the GIT tract is unknown, but most species are anaerobic and metabolize amino acids rather than sugars, suggesting a potential role in protein degradation. An increase in the prevalence of Fusobacteria within the human colon has been linked to the presence of cancer cells (Gao *et al.* 2015), but it is unclear as to whether Fusobacteria are involved in tumor formation or simply make use of tumors as attachment sites for growth. Of relevance to their protein-degrading capability is that an increased prevalence of Fusobacteria has been reported in the microbiomes of vertebrates that commonly feed on carrion (alligators, vultures).

Proteobacteria – As with the Firmicutes and Bacteroidetes, the Proteobacteria are abundant in the GIT of most vertebrates, and these three phyla essentially make up the core vertebrate GIT microbiome. While the Proteobacteria may be the third most abundant phylum in the GIT of a typical mammal, they have been found to be the dominant phylum in the GIT of some fish, reptiles and birds. The Proteobacteria is the largest bacterial phylum in terms of the number of culturable bacteria, and has been the most extensively studied. While all of its members are Gram-negative, they are metabolically diverse and include heterotrophs and autotrophs, with metabolisms including respiration, fermentation, photosynthesis and chemoautotrophy. The Proteobacteria are typically classified into the Alpha-, Beta-, Gamma-, Delta- and Epsilon-Proteobacteria, of which the Gammaproteobacteria are the most common in the vertebrate GIT. These bacteria typically break down and ferment complex sugars, and include the well-studied

bacteria *Salmonella* and *Escherichia*, the latter of which may be important in production of vitamins for the host. Members of the Betaproteobacteria and Epsilonproteobacteria can also inhabit the vertebrate GIT, and the Epsilonproteobacteria includes one genus (*Helicobacter*) that is a natural inhabitant of the mammalian stomach. These and other Epsilonproteobacteria have also been found in the GIT of birds and reptiles.

Tenericutes – The Tenericutes have typically been grouped as the Mollicutes, an unusual group of Firmicutes, but are more correctly identified as their own phylum. They are characterized by a lack of cell wall and are typically of very small physical size and genome size. Many are parasitic (of hosts ranging from plants to vertebrates) and all appear to require a host, making them difficult to culture and therefore study. Within the vertebrate GIT, members of the Tenericutes have been identified as important members of the gut communities of fish and juvenile amphibians, where they may aid in nutrient processing, particularly for detritivorous hosts. They have also been found to be dominant members of the microbiomes of corals (Kellogg *et al.* 2009; Gray *et al.* 2011) suggesting that they may be particularly important for aquatic organisms.

Box 3 – Future Directions

Broader, deeper sampling

Compared to humans, our current knowledge of the microbiomes of other vertebrates, especially non-mammals, is extremely limited. For some anatomical regions we have essentially no information at all on the microbiome present, even for entire vertebrate classes (e.g. the skin microbiome of reptiles). Similarly, there are entire taxonomic groups that have never been sampled for any associated microbial community (e.g. amphisbaenids, sphenodontids). There have been recent calls for broader sampling of the global microbiome (Alivisatos *et al.* 2015; Dubilier *et al.* 2015) and the same effort is needed for non-human vertebrates. If evolutionary and ecological patterns in vertebrate microbiomes are to be examined effectively then a much broader sampling effort is needed. NGS technology has developed to the point where a single microbiome sample can be sequenced for less than US$10 (as of 2016), yielding tens of thousands of 16S rRNA gene sequences. Analyses of hundreds of samples, potentially many different vertebrate species (broad sampling) or many individuals within a species (deep sampling), are therefore affordable for many research groups. Rather, we are at a point where collecting the samples, rather than analyzing them, is the limiting factor. Thus, coordination and cooperation between scientists in different fields is likely to be essential, with field zoologists and ecologists collecting samples for lab-based microbial ecologists and microbiologists, and bioinformatics specialists working with the subsequent data. Sampling is easier if the microbiome can be sampled non-invasively: skin samples can be taken by simple swabbing, and the gut microbiome could be determined from feces (although there are problems with that approach) or cloacal samples for some vertebrates, which we have found to be effective in elucidating differences between individuals (Colston *et al.* 2015). Making microbiome sampling

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a default process when collecting tissue samples from any vertebrate taxa for phylogenetic/phylogeographic studies would require not much more than researchers carrying sterile swabs and tubes into the field, and vastly increase our knowledge of the vertebrate microbiome, as well as potentially link microbiome composition to phylogeny. Additionally, it has become commonplace for natural history collections to store genetic samples (muscle tissue, blood, fin clips, feathers etc) associated with voucher specimens. Many of these samples are stored in ultracool freezers, the same equipment necessary for the storage of microbiome samples, and we propose that the host's microbiome is of no less importance to collect and maintain than tissue samples.

As well as broadening our knowledge of the microbiomes of different vertebrate groups, deeper knowledge in individual to individual variation within species is desperately needed. This will be difficult for many species, for which field surveys may only detect a few individuals, but focusing on common taxa, especially those that can be easily collected en masse (e.g. many fish) would be a place to start. Studies on captive animals could contribute to this area, and while the microbial communities of captive vertebrates may not be a reflection of those in the wild, they do provide a relatively easy mechanism to sample multiple individuals of the same species. It's surprising that most of the microbiome studies on captive vertebrates have still just sampled a limited number of individuals, but greater coordination between different groups could be encouraged (for example, between different zoos or aquariums that house the same species). Sampling the microbial communities associated with many individuals of captive species would help determine the extent of individual variation within a more controlled setting, but also allow us to more clearly elucidate the influence of age, growth rate, diet, and even genetic relatedness on microbiome composition.

The biogeography of the vertebrate microbiome

Concurrent with efforts to sample more broadly and deeply, more extensive sampling across the ranges of species is needed. With a few exceptions, studies on wild vertebrates have tended to focus on animals sampled at one or a few specific locations, so that we have little knowledge of biogeographic patterns in microbiome composition and how much it may be driven by environmental variation or differences in diet or behavior on different parts of the range. Microbial biogeography has emerged as a field in and of itself (Dolan 2005; Martiny *et al.* 2006; Fuhrman *et al.* 2008) but the majority of studies on spatial patterns in microbial community structure or diversity have focused on microbial assemblages in aquatic or terrestrial environments, or on the microbiomes of plants rather than animals. How the gut or skin microbiome varies across the range of a vertebrate host is an intriguing question, particularly for ectothermic organisms which may be at different temperatures in different parts of their range, or for any organism that may shift its diet depending on location or because of seasonal variation in food availability.

The human gut microbiome has been found to be substantially different in different parts of the world (De Filippo *et al.* 2010; Yatsunenko *et al.* 2012), and such differences are likely attributable to diet as studies have typically compared urban individuals in Western countries to agrarian societies elsewhere. In a broad study of microbiomes from over 200 individuals within a single country (USA), the Human Microbiome Project found that geographic location was not a strong factor influencing microbiome composition, and that ethnic/racial origin was a stronger correlate to microbiome structure and function (The Human Microbiome Project Consortium 2012). Thus, genetics would appear to be a stronger influence on the human microbiome than geographical location. How this finding might be extended to other vertebrates that are less

individually mobile and for which individuals within a specific geographic part of the range are likely to be genetically related is unknown.

While advances in technology have made humans more mobile at the individual level than other vertebrates, whole populations of non-mammalian vertebrate species (especially birds) show extensive mobility during migratory events. How the microbiome of such species changes either during migration events or from one (likely seasonal) range to another is largely unknown. In a migratory fish species (Atlantic salmon, *Salmo salar*), gut community composition was influenced by life-cycle stage rather than geography (Llewellyn *et al.* 2015), but this may not be typical given the reproductive basis for salmon migration. Collaboration between scientists in the different ranges of seasonally migratory species will be critical to understand the effects of shifting range on the microbiome, which may reflect changes in environmental influence as well as diet.

Invasive species may present an experimental system to examine biogeographic patterns in the animal microbiome but we know of no studies to date that have compared the microbial communities of vertebrates in their native and expanded invasive range. A few studies on invasive invertebrates have suggested that hosts in native and invaded regions show similar microbiome structure, with only minor differences in community composition (e.g. Bansal *et al.* 2014), but even that area is underexplored. Given the importance of invasive species to issues regarding biodiversity, assessing the relationships between invasive species and their microbiome would seem to be critical.

Functional aspects of the vertebrate microbiome (metagenomics)

Most NGS studies of the microbiomes of non-mammalian vertebrates have focused solely on community composition, typically using partial 16S rRNA gene sequencing to describe

the structure of the gut or skin assemblage. Studies of the human microbiome have incorporated a metagenomics approach to also characterize the functional genes present in the microbiome of different individuals and body habitats. Such studies have found that while the composition of the microbiome changes from body region to body region, or from individual to individual, many microbial metabolic pathways are prevalent across most individuals and body habitats (Human Microbiome Project Consortium 2012, Lozupone *et al*. 2012). The human gut microbiome, for example, always contains the genes for central pathways in carbohydrate and amino acid metabolism, regardless of the individual bacterial species present (Turnbaugh et al. 2009). Such functional similarity might also occur in comparisons between taxa, with, for example, animals that are phylogenetically distant but overlap in diet, potentially having endogenous microbial communities that have similar properties (Hanning and Diaz-Sanchez 2015). Herbivorous mammals that rely on their gut microbiome for cellulose digestion are an obvious example, and comparative metagenomics has been used to compare the functional capability of the gut microbial communities of agriculturally important mammals (Lamendella *et al.* 2011).

The application of functional approaches to the microbiomes of non-mammalian vertebrates is sorely lacking even though techniques such as shotgun metagenomics could be used to answer questions relating to the influences of diet, biogeography, and phylogeny on the host microbiome. Functional comparisons across taxonomic groups that show similar properties, such as between herbivorous fish, reptiles, and amphibians; or between different groups of fasting reptiles; or even between different taxa inhabiting the same environment (e.g. comparing the functions of the skin and gut-associated microbial communities of co-existing amphibians and fish in the same pond) could reveal important findings in regards to how structurally distinct microbial assemblages can show overlapping function. Costs for such metagenomics analyses

exceed those for 16S rRNA gene surveys of community structure, but if care is taken during the initial sampling and DNA extraction process, any microbiome sample taken could be preserved for potential future metagenomic analysis. A greater sampling effort of non-mammalian microbiomes coupled with careful archiving of samples that can be used for multiple levels of analyses, and potentially by different groups, would be highly beneficial.

The influence of the microbiome on host evolution and diversification

Studies of the microbial communities associated with eukaryotic organisms have an overwhelming tendency to emphasize the impacts of host ecology (diet, age, growth rate, location, genetics) on the microbiome. The reverse – the influence of the microbiome on the ecology and evolution of the host – has rarely been considered. Research on the human microbiome is beginning to elucidate the role of our associated microbial community in our physiology, immunity, and even neurological and thought processes (Cho & Blaser 2012; Foster *et al.* 2013; Lyte 2013; Stilling *et al.* 2014)). As such, it's becoming clear that the ecology of the host is highly dependent on their associated microbiome, and that the host and microbiome are likely in a hologenomic state of coevolution to maximize the success of both (Brucker & Bordenstein 2012; Amato 2013). But to what extent does the microbial community associated with vertebrates actually drive diversification?

Addressing such a question will require more extensive analyses of the microbiomes of closely related species, as well as of analyses of differing individuals within a species (essentially, the broader, deeper sampling that we highlight above). For example, surveying the gut microbial communities associated with a broad taxonomic range of vertebrates such as the squamate reptiles or the perciform fish, and linking the composition of the microbiome to ecological factors such as diet or the environment as well as to evolutionary phylogeny and

diversification rate may help understand whether the endogenous microbial community can itself be thought of as an ecological trait; potentially influencing both host diversification and community assembly. Dietary changes can reflect shifts in the niche, one of the classic traits used to characterize ecological processes affecting speciation (Kozak & Wiens 2010; Jeraldo *et al.* 2012), and clearly affects, and is potentially affected by, the gut microbial community. Similarly shifts in parity mode are associated with diversification in reptiles (Sites *et al.* 2011; Pyron & Burbrink 2014), and may represent another ecological trait that influences or is influenced by the endogenous microbiome. To what extent changes in microbiome structure have been coupled with or even driven diversification patterns in different branches of the vertebrate tree of life is an intriguing question for future work.

Acknowledgements

CHAPTER II

PHYLOGENETIC ANALYSIS OF BACTERIAL COMMUNITIES IN DIFFERENT REGIONS OF THE GASTROINTESTINAL TRACT OF *AGKISTRODON PISCIVORUS*, THE COTTONMOUTH SNAKE

ABSTRACT

Vertebrates are metagenomic organisms in that they are composed not only of their own genes but also those of their associated microbial cells. The majority of these associated microorganisms are found in the gastrointestinal tract (GIT) and presumably assist in processes such as energy and nutrient acquisition. Few studies have investigated the associated gut bacterial communities of non-mammalian vertebrates, and most rely on captive animals and/or fecal samples only. Here we investigate the gut bacterial community composition of a squamate reptile, the cottonmouth snake, *Agkistrodon piscivorus* through pyrosequencing of the bacterial 16S rRNA gene. We characterize the bacterial communities present in the small intestine, large intestine and cloaca. Many bacterial lineages present have been reported by other vertebrate gut community studies, but we also recovered unexpected bacteria that may be unique to squamate gut communities. Bacterial communities were not phylogenetically clustered according to GIT region, but there were statistically significant differences in community composition between regions. Additionally we demonstrate the utility of using cloacal swabs as a method for sampling snake gut bacterial communities.

INTRODUCTION

Vertebrates are metagenomic organisms; they are not only composed of their own genetic material, but also that of their associated microbial communities (Ley *et al.* 2009). The majority of these microorganisms are found in the host intestinal tract, and presumably assist in essential processes of energy and nutrient acquisition(Ley *et al.* 2008a). The ecological and evolutionary forces that act on both the host and it's trillions of resident microorganisms sculpt the endogenic microbiome. With the advent of next generation sequencing technologies we are now better able than ever to characterize this observed microbial diversity. However, most studies investigating evolutionary patterns in vertebrate gut microbiomes have focused on mammals (Ley *et al.* 2008a, 2009) and even among these studies, many have used captive animals from zoos or farms rather than wild populations. Very few studies have examined the gut microbiome of squamate reptiles (snakes, lizards), despite this being one of the most diverse and successful vertebrate clades.

The cottonmouth (*Agkistrodon piscivorus*, Serpentes, Viperidae) is a semiaquatic snake widespread throughout southeastern United States. The ecology and demographic history of *A. piscivorus* has been well studied, and it is often used as a model system in studies of venom evolution (Vincent *et al.* 2004a; Roth 2005; Guiher & Burbrink 2008; Lomonte *et al.* 2014). Though *A. piscivorus* is considered a generalist predator, preying upon reptiles, birds and invertebrates, the diet is dominated by amphibians and fish (Ford 1997; Vincent *et al.* 2004a). The paucity of information on the microbiome of wild vertebrate gastrointestinal tract (GIT) regions renders studies of any species meritorious, and exploration of the gut microbiome of *A. piscivorus* is particularly interesting as almost all other aspects of its ecology and biology are well known. Given the extent of knowledge on this organism's natural history, inferences regarding factors influencing the composition of its GIT microbial community should be possible

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once that community has been well characterized. In this study we examine the bacterial communities of the small and large intestines, and cloaca of eight individuals of adult *A. piscivorus*. Our results reveal the presence of distinct bacterial community composition in each GIT region, provide novel insights into the diversity of squamate reptile associated bacterial communities in the wild, and demonstrate the utility of using non-lethal cloacal swabs to sample this diversity.

MATERIALS AND METHODS

Ethics Statement

Adult snakes were collected and sacrificed in accordance with IACUC protocols approved by the Committee for Animal Care and Use at the University of Mississippi (#13-02 & #13-04). Euthanasia was performed using an overdose of the anesthetic lidocaine, injected into the brain (UM IACUC SOP #13-02). Collecting permits were obtained from the Mississippi Department of Wildlife, Fisheries, and Parks (permit #'s 0827101 & 1009112).

Microbial Sampling

Snakes were sampled from two sites in Winston County (one individual, N32.98463 X W088.9980) and Lafayette County (seven individuals, N34.427238 X W089.38631), Mississippi, in spring of 2011 and spring of 2012 (Table 1). All snakes sampled were encountered during nighttime surveys along small streams leading to larger bodies of water. Snakes were collected by hand and safely restrained with clear plastic tubing placed over the head during sample collection. Once restrained, snakes were palpated to evaluate whether prey items were present in the GIT then the exterior cloaca of the snake was cleaned using a sterile alcohol pad. This sterilization step was to ensure that the cloacal sample primarily included cloaca associated microbes rather than environmental or transient microbes. Following cleaning,

cloacal swabs were collected by inserting a sterile polyester-tipped applicator (Fisher Cat# 23-400-122) into the cloaca, taking care not to insert beyond the coprodeum and into the large intestine, then turning the swab several times before withdrawing. Once withdrawn the applicator was immediately placed into a sterile 2 ml tube and placed on ice before being transferred to a -20°C freezer prior to DNA extraction.

Table 1. Voucher numbers, locality information and region sampled for *Agkistrodon piscivorus* used in this study.

TJC Field ID #	OMNH Catalog #	County	Small Intestine	Large Intestine	Cloaca
103	44090	Winston	X	X	X
110	44088	Lafayette	X	X	X
111	44089	Lafayette	X	X	X
122	-	Lafayette	-	-	X
123	-	Lafayette	-	-	X
124	-	Lafayette	-	-	X
125	-	Lafayette	-	-	X
130	-	Lafayette	-	-	X

Three individuals (samples 103, 110, and 111) were sampled in more detail to determine the bacterial communities of their small and large intestines. These snakes were transported to the Department of Biology at the University of Mississippi where they were humanely euthanized. Immediately following death, a mid-ventral incision was made to expose the GIT, which was then removed. None of the individuals had identifiable prey items present in the GIT. Incisions were made in proximal and distal ends of both the small and large intestines, which were then swabbed with sterile polyester-tipped applicators that were immediately placed in sterile 2 ml collection tubes and frozen (-20°C) until DNA extraction. The remainder of the snake was preserved in 10 % buffered formalin and whole specimens were deposited at the Sam Noble Oklahoma Museum of Natural History (Table 1).

DNA Extraction

Microbial DNA was extracted by a bead beating procedure using MoBio Power Soil Extraction kits (MoBio Laboratories, Carlsbad, CA, USA). We followed the manufacturer's standard DNA extraction protocol with minor adjustments. Thawed applicator tips were placed into bead tubes containing lysis buffer, and 50-100 µl of the lysis buffer was used to rinse any remaining particles that may have become dislodged from tips out of the 2ml collection tube and into the bead tube. Additional adjustments included incubating samples at 65°C for 15 min after the addition of Solution C1, and vortexing bead tubes horizontally for 25 min.

PCR Amplification and Analysis

We used a nested PCR approach and bacterial specific primers to amplify a variable region of the 16S rRNA gene for initial analysis of the gut bacterial community by denaturing gradient gel electrophoresis (DGGE). This nested approach was necessary as DGGE can only be performed on fragments <500bp, and our template DNA was of low quantity rendering direct amplification of small fragments difficult. We first amplified near full-length fragments of the bacterial 16S rRNA gene using primer sets Bac8f and Univ1492r, and used this as our template for amplifying a shorter region for use in DGGE with the primer sets Bac1070f and Univ 1392GCr. Primers, PCR protocols, and cycle conditions have been previously described (Jackson *et al.* 2001b). Amplification products from the second round of amplifications (bases 1070-1392) were analyzed in DGGE gels using a 40% to 70% denaturant gradient, and electrophoresis for 20 h at 80 V. Following electrophoresis, gels were stained with SYBR Green I and visualized by UV transillumination using a Kodak Gel Logic 200 system running Molecular Imaging Software 4.0 (Eastman Kodak, Rochester, NY, USA). Banding patterns were converted to binary data based on presence or absence of specific bands in each sample. Binary data were then used to

create a distance matrix, showing similarity between samples (Yue & Clayton theta index (Yue & Clayton 2005)), and these relationships were visualized by ordination of samples through non-metric multidimensional scaling (NMDS). All analyses were performed using the bioinformatics software Mothur (Schloss *et al.* 2009).

Pyrosequencing

DGGE analyses revealed that the proximal and distal ends of the large and small intestines possessed near identical banding patterns, therefore we combined the DNA extraction from these regions such that each dissected individual had a single small and single large intestinal sample analyzed by pyrosequencing, along with the cloacal samples from both dissected and released individuals. The initial (bp 8-1492) 16S rRNA amplicons from each sample were sequenced using bacterial tag-encoded FLX amplicon 454 pyrosequencing (bTEFAP) (Dowd *et al.* 2008) at the Research and Testing Laboratory sequencing facility (Lubbock, TX). Library amplification was performed with the bacterial primers 939f and 1392r (Jackson *et al.* 2001b; Baker *et al.* 2003) under the following conditions: 95°C for 5 minutes, followed by 35 cycles at 95 °C for 30s, 54 °C for 40s, an extension at 72 °C for 1 minute, followed by a final elongation of 10 minutes at 72°C. Sequencing was performed on a Roche 454 FLX titanium instrument using standard reagents and following manufacturer's guidelines. Sequences were deposited in the NIH NCBI Sequence Reads Archive (SRA Accession numbers SAMN03287547-SAMN03287560).

Sequence Analysis

Pyrosequence data was accessed and processed in the program Mothur (Schloss *et al.* 2009) following general procedures recommended by Schloss et al. (Schloss *et al.* 2011). Following denoising and barcode removal, sequences were aligned using the greengenes reference database

(http://greengenes.secondgenome.com/, May 2013 version) and sequences differing by only a single nucleotide were grouped together. Sequences were checked for chimeras using the chimera.slayer command in Mothur and potential chimeras were removed. The resulting sequences were classified according to the greengenes database and any non-bacterial 16S sequences were removed. The remaining sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity.

The distribution of OTUs in each sample was used for analyses of diversity and comparisons of community structure. Because samples varied in the number of final valid sequences obtained, all analyses were performed on a randomly sampled subset of the total dataset for each sample, which corresponded to the number of sequences in the smallest sample (i.e. all samples were standardized to be equivalent to the sample with the lowest number of valid reads). This random subsampling was performed 1,000 times for each analysis, with the composite outcome reported. Alpha diversity within each sample was determined by Schao and inverse Simpsons indices, and rarefaction and collection curves were used to visualize whether our procedures included enough sampling (enough reads) to assess this diversity. Beta diversity (comparisons of bacterial community structure between samples) was examined using the Yue & Clayton theta similarity index (Yue & Clayton 2005) which accounts for proportional abundance of OTUs in a sample. Similarity between samples was visualized by NMDS, Venn diagrams, as well as dendrogram construction. We tested the spatial separation of samples observed in NMDS through analysis of molecular variance (AMOVA) and analysis of similarities (ANOSIM). Dendrograms were constructed based on Yue & Clayton theta (thetayc) distances and a 95% majority rule consensus tree was generated from the distribution of 1000 trees without burn in using the program TreeAnnotator (Rambaut & Drummond 2007).

RESULTS

DGGE Analyses

While based on a limited number of samples from just two locations, NMDS of community

similarity based on DGGE binary data showed a clear pattern of distinct small intestine, large

intestine, and cloacal communities, with only slight overlapping of multidimensional space

between the large and small intestine samples (Figure 1A). There was no apparent association in

GIT bacterial community structure among individuals or localities, although our ability to test

this was limited by the number of individuals sampled. All regions of the GIT show similar

levels of diversity and richness as inferred from DGGE banding patterns. Band number, used as

a proxy for richness (Jackson *et al.* 2001a), ranged from 8-29, with a mean of 17.

Sequence Analyses

After alignment, gap removal, and potential chimera removal we recovered 40,317 valid

sequence reads, representing 7,137 unique sequences with a median length of 267 bp from our

pooled dataset. The classified sequences were binned into OTUs and rarefaction suggested that

we recovered >98% of the diversity in all but one of our samples, and with that sample

(individual 103l) we recovered 95% of the diversity (Table 2).

Table 2. Statistical analyses of bacterial 16S rRNA gene pyrosequence data obtained from the gastrointestinal tract (GIT) of individual (ID) *Agkistrodon piscivorus* **(numbered 103-130).**

Different regions of the GIT are designated as (c) cloaca (l) large intestine (s) small intestine. Inverse Simpson's diversity index and Schao were used to assess alpha diversity and compare species diversity between samples. S_{obs} = observed number of species.

ID	103 (c)	103 (l)	103 (s)	110 (c)	110 (l)	110 (s)	111 (c)	111 (l)	111 (s)	122 (c)	123 (c)	124 (c)	125 (c)	130 (c)
Coverage	99.22%	95.45%	99.03%	98.91%	99.49%	99.64%	99.14%	99.26%	99.70%	98.31%	99.02%	98.92%	99.26%	99.17%
S_{obs}	40	143	52	28	31	15	68	51	110	64	63	55	54	29
S_{Chao1}	63.8	208	80.9	58.3	109	29	105.5	59.23	161.7	88.4	84.4	76.9	67.2	32.5
Inverse Simpsons index	4.575	11.559	4.471	2.497	3.069	1.085	10.489	7.231	13.940	8.599	8.983	7.918	5.961	4.448

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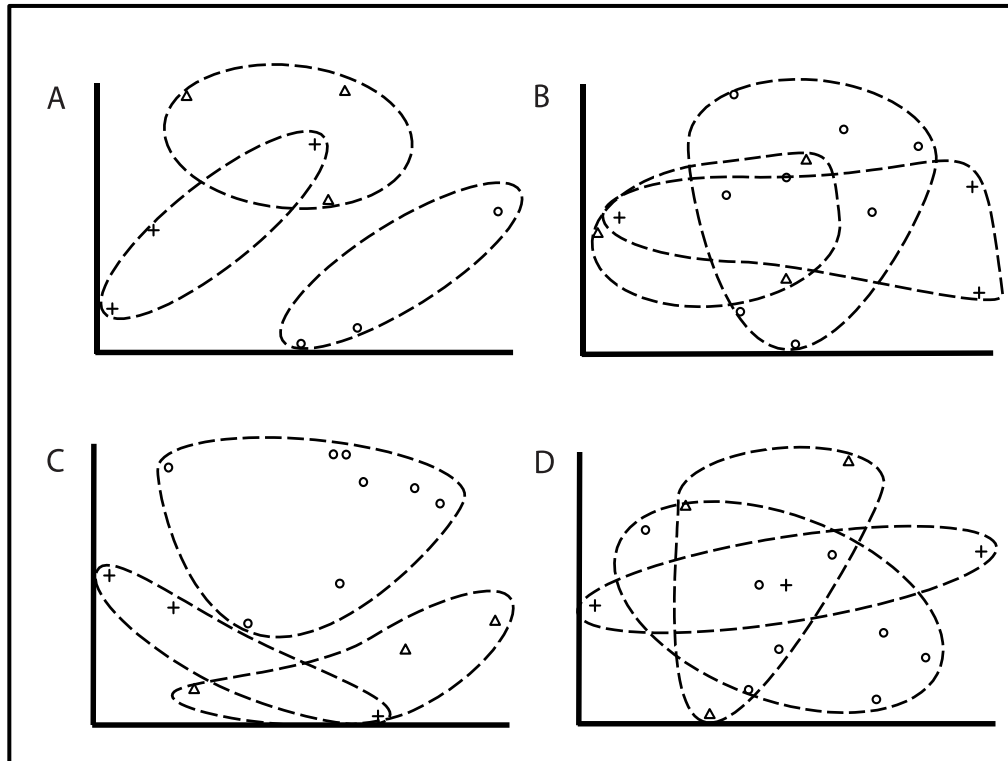
Figure 1. Nonmetric multidimensional plots of bacterial communities.
Nonmetric multidimensional plots (three dimensions, stress <0.2; the first two dimensions are shown) based on Yue & Clayton theta (thetayc) similarities of bacterial communities from the small intestine (triangles), large intestine (plus signs), and cloaca (circles) of *Agkistrodon piscivorus*. Plots are determined from: A) DDGE profiles, B) all OTUs recovered from 454 sequencing, C) dominant OTUs recovered from 454 sequencing (rarest 1% of sequences removed), D) very dominant OTUs recovered from 454 sequencing (rarest 10% of sequences removed). Dashed ellipses indicate groupings based on region of the GIT.

Bacterial diversity recovered in our 7,137 unique sequences was binned into 503 distinct OTUs spanning 14 bacterial phyla, with <0.002% (just 92 sequences) of the 40,317 sequence dataset designated as "unclassified Bacteria". Among individuals for which all three regions were sampled the large intestine harbored the most diverse bacterial communities in two of the three individuals (Table 2). In terms of community composition, the large intestine samples were dominated by sequences affiliated with the Bacteroidetes, followed by Firmicutes, Proteobacteria, and Lentisphaerae, while members of the Proteobacteria were the dominant

group in both the small intestine and cloaca samples, followed by sequences classified as Firmicutes and Bacteroidetes (Figure 2). Gammaproteobacteria were the dominant subphylum of Proteobacteria in all but two samples; one small intestine (110) sample and one cloacal sample (111) had Deltaproteobacteria and Betaproteobacteria, respectively, as their dominant Proteobacteria. Importantly, the dominant bacterial phyla found in both the small and large intestines were also found in cloacal samples (Figure 2).
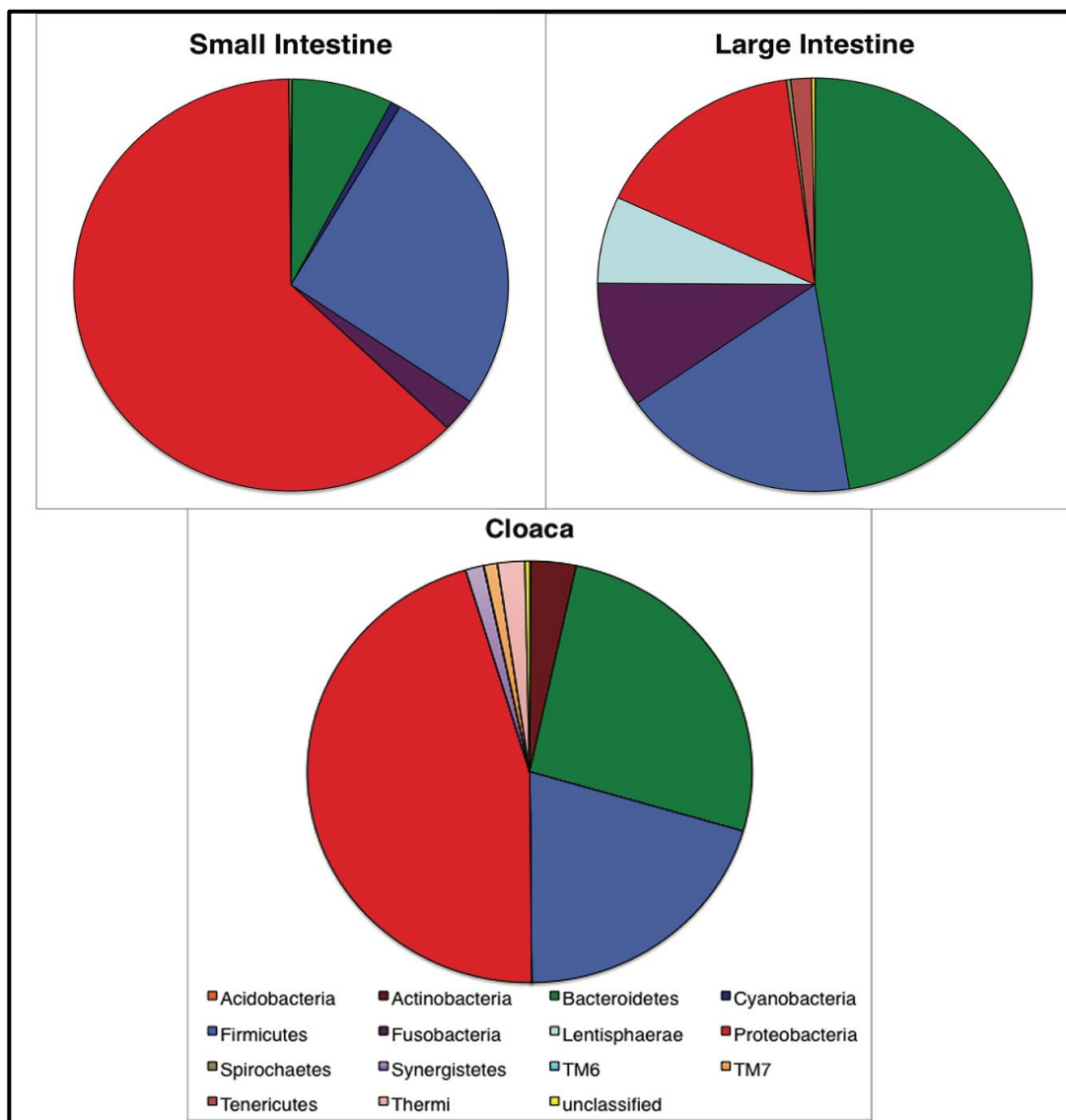
Figure 2. *Agkistrodon piscivorus* GIT bacterial abundance.
Relative abundance of major bacterial lineages found in various regions of the gastrointestinal tract of all *Agkistrodon piscivorus* as identified 16S rRNA gene pyrosequencing. Proportions

represent the proportion of 454 sequence reads classified as being in that taxon (number of reads ranges from 5,620-14,905).

NMDS plots based on pyrosequence data did not show as tight of a grouping of samples by GIT region as was produced by DGGE analyses (Figure 1A, B). ANOSIM and AMOVA tests of spatial separation did suggest a significant difference in community composition among all three regions as a group (AMOVA: df=2 F_s=1.902 p=0.005, ANOSIM: R^2=0.606 p=0.001), but none of the individual pairwise comparisons between different GIT regions were significant. Next generation sequencing is likely to detect ultra-rare species that are typically not detected by DGGE (Caporaso *et al.* 2011), and these may not have an important role in the GIT community, or could represent transitory cells that are not permanent members of the gut community. Therefore we also analyzed the pyrosequence data after sequentially removing the rarest 1%, 5% and 10% of sequences. NMDS of communities with the rarest 1% and 5% of sequences removed resembled the structuring seen in our DGGE data, although that signal was lost once the rarest 10% of sequences are removed (Figure 1C (1%), D (10%), 5% removed not shown in figure for clarity). AMOVA following the removal of the rarest 5% of sequences still suggested overall community differences among GIT regions (p=0.007), without any individual pairwise comparisons between GIT regions being significant. However, ANOSIM did detect significant differences for all pairwise comparisons of GIT regions once any level (1%, 5%, or 10%) of the rarest sequences was removed (Table 3).

Table 3. Results of ANOSIM analyses comparing the bacterial communities in different regions of the GIT of individuals of *Agkistrodon piscivorus*. GIT regions are the large intestine (L), small intestine (S), cloaca (C). Analyses were performed with different levels of the rare sequences removed (<1%, <5%, or <10% of total reads).

Region	1% Removed		5% Removed		10% Removed	
	R^2	p	R^2	p	R^2	p
C-L-S	0.614	0.001	0.469	0.002	0.441	0.005
C-L	0.683	0.006	0.524	0.012	0.511	0.012
C-S	0.718	0.006	0.551	0.011	0.546	0.011

Because we had cloacal samples from both dissected and released specimens, we focused on the cloaca as our primary GIT region of interest, as this would allow future sampling to avoid sacrificing individuals. Furthermore, of the 22 OTUs representing greater than 1% of the sequence reads, all but four were found in the cloaca as well as other regions (Table 4). Based on proportions of sequence reads obtained, the most abundant bacterial lineage in the cloaca varied among individuals: Members of the Bacteroidetes were dominant in two samples, Proteobacteria (primarily subphyla Gamma and Beta) were dominant in two, similar levels of abundance of Bacteroidetes and Firmicutes were found in three, while one sample had similar proportions of Bacteroidetes, Proteobacteria and Firmicutes (Figure 3). At a finer taxonomic scale, the most abundant sequences affiliated with Bacteroidetes were classified as members of the genus *Bacteroides,* with two different species dominating in the large intestine and cloaca (Table 4). While the most abundant Proteobacteria (specifically Gammaproteobacteria) sequences obtained from the cloaca were classified as either *Salmonella enterica*, or as unclassified Enterobacteriaceae, those of the small intestine were identified as *Pseudomonas veronii* and Aeromanadaceae (Table 4). Both *Enterobacter* and *Acinetobacter* were found in all regions of the GIT. The most numerous Firmicutes sequences were in the family Clostridiaceae and those we were able to classify to genus were *Clostridium* (Table 4).
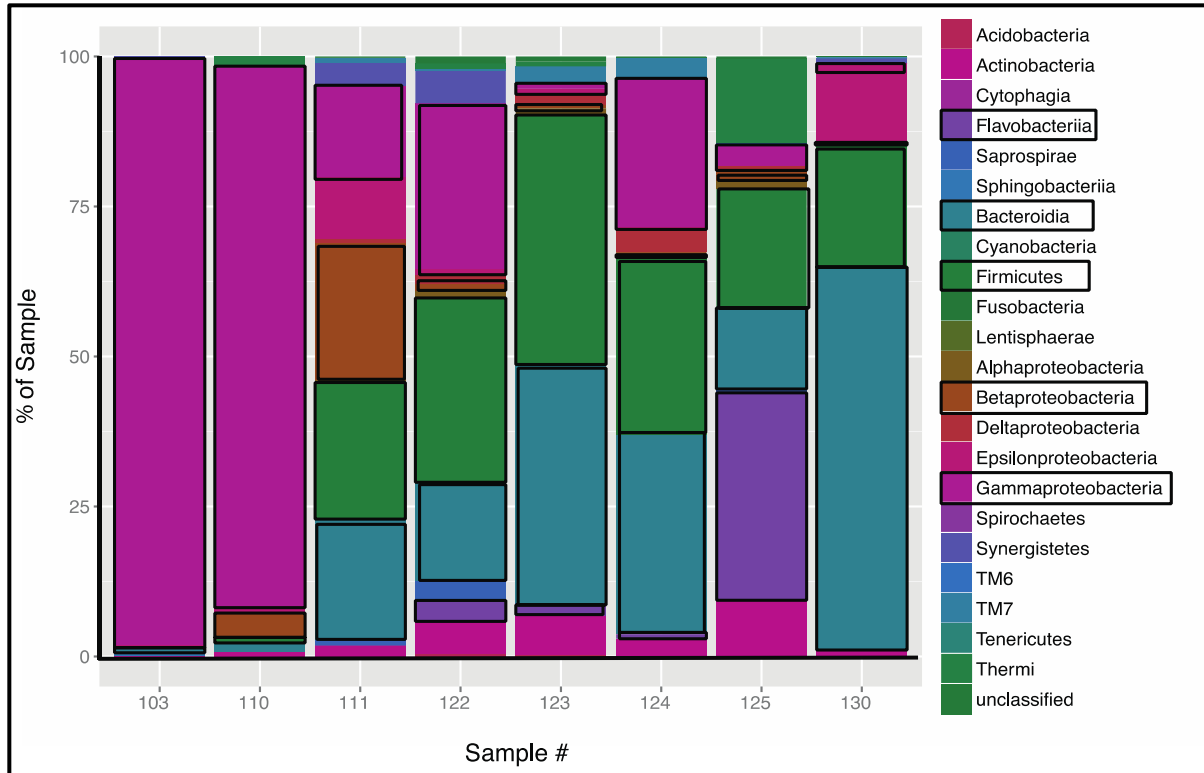
Figure 3. Bacterial abundance present in the cloaca of *Agkistrodon piscivorus*.
Relative abundance of bacterial lineages found in cloacal samples of *Agkistrodon piscivorus* individuals (numbered) as identified 16S rRNA gene pyrosequencing. Abundances are based on proportional number of 454 reads (from 839 – 2919) total reads per sample). Major bacterial groups are outlined by black boxes.

Table 4. Distribution and classification of 16S rRNA-defined bacterial OTUs representing >1% of the total proportion of reads obtained from the GIT of individuals of *Agkistrodon piscivorus*. Region designates the region of the GIT where that OTU was detected: large intestine (L), small intestine (S), cloaca (C).

Phylum/Subphylum	Finest Taxonomic Classification	% of total	Region
Bacteroidetes	*Bacteroides sp.*	10.07	LS
Gammaproteobacteria	Enterobacteriaceae	7.50	LSC
Deltaproteobacteria	Desulfovibrionaceae	6.40	LS
Gammaproteobacteria	Enterobacteriaceae	5.34	LSC
Bacteroidetes	Porphyromonadaceae	5.28	LSC
Gammaproteobacteria	*Salmonella enterica*	4.61	LSC
Gammaproteobacteria	*Pseudomonas veronii*	4.23	LSC
Gammaproteobacteria	Aeromonadaceae	3.25	LSC
Fusobacteria	*Cetobacterium somerae*	3.18	LSC
Firmicutes	Clostridiaceae	2.99	LSC
Firmicutes	Peptostreptococcaceae	2.98	LSC
Gammaproteobacteria	*Enterobacter sp.*	2.37	LSC
Gammaproteobacteria	*Acinetobacter sp.*	2.00	LSC
Firmicutes	*Clostridium sp.*	1.87	LSC
Firmicutes	Clostridia	1.79	C
Betaproteobacteria	*Janthinobacterium lividum*	1.63	S
Gammaproteobacteria	*Edwardsiella sp.*	1.56	S
Bacteroidetes	Weeksellaceae	1.54	C
Bacteroidetes	*Bacteroides sp.*	1.21	C
Epsilonproteobacteria	*Campylobacter fetus*	1.14	C
Gammaproteobacteria	Enterobacteriaceae	1.07	LSC
Betaproteobacteria	*Achromobacter sp.*	1.06	C

Venn diagrams revealed that some OTUs in each region of the GIT were shared by all three individuals sampled, but the majority of OTUs in each region were unique to a given individual (Figure 4). Dendrogram analysis did not reveal a definitive pattern of clustering by GIT region sampled or individual, although cloacal samples tended to group together (Figure 4).
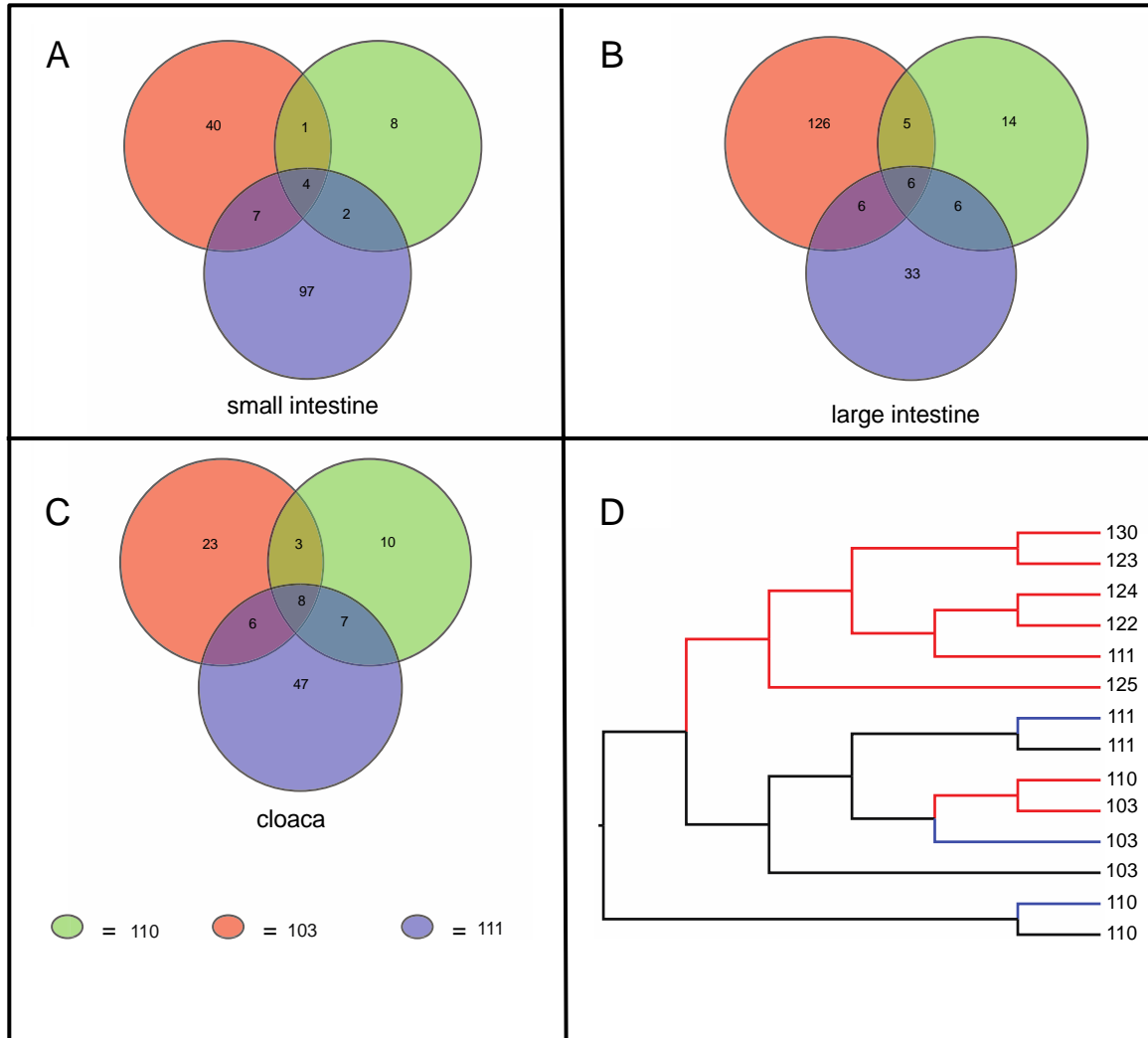
Figure 4. Bacterial community similarity by region and phylogenetic reconstruction of bacterial communities found in Agkistrodon piscivorus.

Graphical representation of bacterial community similarity in GI regions sampled from *Agkistrodon piscivorus* individuals: A- C) Venn diagrams of small intestine (A), large intestine (B) and cloacal (C) samples from the three individuals sampled destructively (103, 110, 111). Circles are drawn such that the area of the circle is proportional to the number of OTUs found in each region. Numbers represent the number of OTUs either shared or specific to that individual. D) 95% majority rule consensus tree based on Yue & Clayton theta distances for bacterial communities in all individuals sampled (numbered) with branches colored by sample region (blue = small intestine, black = large intestine, red = cloacal).

DISCUSSION

The aim of this study was to characterize the gut microbiome of a non-captive squamate reptile species. Only one other study has examined bacterial community structure in the GIT of wild snakes. That study included two individuals of *A. piscivorus,* the species studied here, but the authors focused on the most abundant bacterial populations in the gut community as determined from DGGE followed by traditional Sanger sequencing (Hill III *et al.* 2008), rather than incorporating the high throughput sequencing approach that we used. Despite the difference in methodology, that study also found the Firmicutes and Bacteroidetes to be the most dominant phyla in the GIT of *A. piscivorus,* although no samples of the cloaca were taken and, presumably because of methodological constraints, the study did not attempt to assess diversity or abundance (Hill III *et al.* 2008). Our study demonstrates the utility of both standardized sampling via cloacal swabs and the greater information in community composition that can be obtained by using next generation sequencing rather than traditional methods of bacterial community analyses.

Both DGGE and next generation sequencing recovered distinct bacterial communities corresponding to discrete regions of the GIT (small intestine, large intestine, cloaca), although this was only apparent when the more common sequence types were considered. As might be expected given the cloaca's terminal location, cloacal samples captured the bacterial diversity found in earlier regions of the GIT, but not necessarily the proportional abundance of specific taxa. While the dominance of members of the Bacteroidetes in the large intestine is consistent with other findings in snakes (Hill III *et al.* 2008; Costello *et al.* 2010), the dominance of members of the Proteobacteria in the small intestine and cloaca was unexpected. Previous work has suggested that the small intestine of captive snakes is dominated by members of the Firmicutes and Bacteroidetes, regardless of physiological processes (e.g. active digestion)

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(Costello *et al.* 2010). Our findings suggest this may not be the case in wild snakes. It is well known that the mammalian gut is dominated by members of the Bacteroidetes and Firmicutes (Ley *et al.* 2008b) and the limited amount of previous work on wild reptiles (e.g. marine iguanas) and captive snakes suggests that this is the same for reptiles (Costello *et al.* 2010; Hong *et al.* 2011). However, a recent meta-analysis compared the GIT bacteria of insects, birds and mammals and found that the bacterial community in insects and birds is more likely to be dominated by Proteobacteria and Firmicutes rather than Bacteroidetes (Hird *et al.* 2014). The dominance of Proteobacteria in the GIT of snakes in this study suggests that predatory snakes may show more similarities to birds in terms of their GIT bacterial communities than to other vertebrate organisms.

In one of the few previous studies to investigate bacterial community structure in wild squamates, Hong *et al.* (2011) found that sequences affiliated with the Firmicutes dominated fecal samples from both marine and land iguanas in the Galapagos, with the classes Bacteroidia and Clostridia being dominant in both host species (Hong *et al.* 2011). They suggested that the similarity between these herbivorous reptiles to mammals in the general composition of their GIT bacterial assemblages is because of the prevalence of herbivory in mammals, and that abundance of members of the Bacteroidetes and Firmicutes in the gut aids in the breakdown and nutrient acquisition of complex polysaccharides (Ley *et al.* 2008a; Hong *et al.* 2011). Wood eating fish species have also been shown to contain similar GIT diversity to herbivorous mammals (McDonald *et al.* 2012) and these similarities (at the level of phylum) have led some authors to suggest that fish served as the original vertebrate hosts to these bacterial communities (Sullam *et al.* 2012; Clements *et al.* 2014). Although *A. piscivorus* is a predatory species (no snakes are herbivorous), Clostridia were the dominant Firmicutes recovered in our analysis,

possibly reflecting a broader function of this subset of Firmicutes in the GIT that is not restricted to herbivory. It may be that this class of obligate fermentative bacteria is so well adapted to the anoxic conditions of the intestines that they are likely to be dominant in the GIT of any larger organism. Cottonmouth snakes are generalist predators and are known to feed on carrion; and some authors suggest that certain populations of *A. piscivorous* my acquire food primarily by scavenging (Devault & Krochmal 2002). Many Clostridia are associated with the fermentation of animal material and this may convey benefits to host species that prey on or scavenge other vertebrates.

All of the individuals sampled in our study were adults, and no detectable prey items were found in dissected individuals or through palpitation of non-dissected individuals. That said, the broad variation in dominant bacterial OTUs we recovered from cloacal samples leads us to suspect that abundance may be influenced by active digestion of prey items that were undetected. Although the bacterial species present in the GIT has been shown to remain constant throughout digestion and subsistence in captive snakes, the relative abundance of these species can be correlated with active physiological processes such as digestion (Costello *et al.* 2010). An additional factor relating to diet variation in the gut bacterial community that has yet to be explored is ontogenetic change. Diet has been shown to change through ontogeny in *A. piscivorus* (Vincent *et al.* 2004b), and it's not unreasonable to suspect that this could lead to changes in bacterial community structure. Acquisition of a new diet has been hypothesized to be a fundamental driver for species' diversification and concomitant gut microbiota evolution (Ley *et al.* 2008b) and dietary changes during ontogeny should also drive changes in gut microbiota. Lastly, it has been shown that in mammalian hosts which undergo torpor during hibernation both bacterial community composition and richness vary dramatically during hibernation (Dill-

McFarland *et al.* 2014). It would be interesting to explore whether this holds true for ectothermic species that undergo hibernation, which *A. piscivorus* does in certain areas of its range.

Our study lays the groundwork for further investigation of gut bacterial community structure in squamate reptiles. We identified discrete bacterial communities that correspond to regions along the GIT and show that cloacal samples encompass the breadth of bacterial diversity found in the snake gut. Additionally we show the utility of our standardized sampling method using cloacal swabs. Interestingly, and in contrast to previous investigations of snakes, we find that at the phylum level the snake gut microbiome shows more similarities to that of birds than to other vertebrates. Future studies should include broader sampling of host species for more detailed comparative analyses, and test whether gut bacterial communities function as either evolutionary or ecological traits.

Acknowledgements

CHAPTER III

EVOLUTION OF THE SQUAMATE REPTILE GUT MICROBIOME

Original Article (to be submitted to *Nature*)

Title: Evolution of the Squamate Reptile Gut Microbiome

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INTRODUCTION

Our understanding of the biology and ecology of metazoans has increased significantly through research into the complex communities of microbes—the "microbiome"—living in, or on, multicellular hosts (Ley *et al.* 2009; Consortium 2012; Alivisatos *et al.* 2015). Microbiome research has fundamentally changed how we approach questions in biology, and we now view animals as *metagenomic* organisms that are not only composed of their own genetic material, but also that of their associated microbial communities (Ley *et al.* 2008a). Microorganisms in the host's intestinal tract are critical to essential processes of energy and nutrient acquisition (Ley *et al.* 2008a), may play a large role in immune response (Berg 2009; Gerritsen *et al.* 2011; Hoyt *et al.* 2015), and investigations of animal function and development need to be rooted in organismal microbiology (Dubilier *et al.* 2015).

Multicellular eukaryotes have interacted with microbes for nearly one billion years, and such interactions likely played a role in vertebrate evolution and speciation (Kardong 1998; Ley *et al.* 2009), contributing to the evolutionary trajectories of entire vertebrate communities (Zilber-Rosenberg & Rosenberg 2008; Ley *et al.* 2009; Fraune & Bosch 2010). Elucidating the role that the microbiome plays in organismal and functional evolution of the host is critical (Waldor *et al.* 2015), although this remains poorly understood. Theory suggests that the ecological and evolutionary forces that act both on the host and its resident microorganisms shape the endogenous (gut) microbiome, with evidence supporting the hypothesis that

microbiomes reflect correlated evolutionary signatures of their host (Ley *et al.* 2009; Phillips *et al.* 2012; Hird *et al.* 2015a).

Among vertebrates, the gut microbiome has been shown to co-evolve and vary with host species, host dietary shifts, and geography (Ley *et al.* 2009; Muegge *et al.* 2011; Phillips *et al.* 2012). These microbiomes differ significantly from free-living microbial communities, play an important role in physiological processes of the host, are linked to adaptation in highly specialized species, and may confer an adaptive advantage to hosts experiencing rapid environmental change (Ley *et al.* 2008a, 2009; Wei *et al.* 2014; Alberdi *et al.* 2016). The importance of understanding the microbiome and its function at all scales, local to global, cannot be understated and this recognition has led to international efforts (Alivisatos *et al.* 2015; Dubilier *et al.* 2015) to increase the diversity of microbiome data collected and infrastructure supporting research in this area (Handelsman 2016).

Most studies investigating host-microbiome evolutionary patterns in non-human vertebrate gut microbiomes have focused on captive animals from zoos rather than wild populations and little is known of the gut microbiome of squamate reptiles (snakes, lizards), despite this being one of the most diverse and successful vertebrate clades (Colston & Jackson 2016). Extant squamate reptiles are found on every continent except Antarctica, occupy a diverse array of niches, and have experienced rapid radiations characterized by accelerated speciation coupled with shifts in niche or parity mode (Kozak & Wiens 2010; Pyron *et al.* 2013; Pyron & Burbrink 2014). To what extent are shifts in niche or parity mode coupled with, driven by, or limited by changes in microbiome structure? To what extent have changes in microbiome structure been coupled with or driven diversification patterns? Few studies have attempted to address these questions. The few published squamate endogenous microbiome studies have

focused on single taxa–and again primarily utilized captive, often lab-raised, animals raised rather than communities from natural populations (Ezenwa *et al.* 2012; Colston & Jackson 2016; but see Colston *et al.* 2015; Kohl *et al.* 2016). To understand the role of the microbiome in squamate evolution it is first necessary to characterize the microbiome in a wide variety of squamate hosts, and from wild rather than captive individuals.

Herein we assemble the largest dataset to date of non-mammalian vertebrate microbiomes. Using 491 field collected gut bacterial samples, spanning 25 families and 142 species of squamate and non-squamate reptiles across three continents, we characterize the "core" microbiome of squamata and use these data to test whether shifts in gut microbial community composition and structure are accompanied by shifts in host niche known to significantly impact diversification rate (Figure 1).
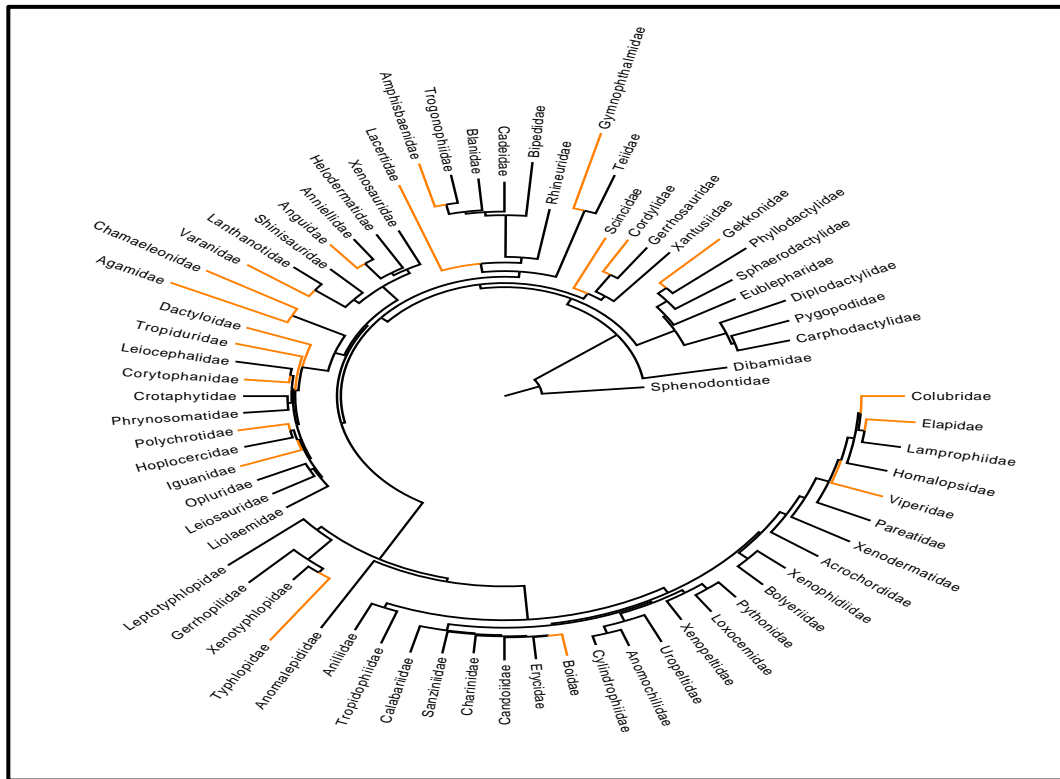
Figure 1. Family-level phylogeny of squamata based on Pyron *et al.* (2013). Highlighted branches indicate families represented by microbiome samples in the present study.

METHODS

Ethics Statement

All animals were collected and sacrificed in accordance with IACUC protocols approved by the Committee for Animal Care and Use at the University of Mississippi (#13-02 & #13-04). Animals were either sampled using minimally invasive methods (Colston *et al.* 2015b) and then released, or euthanized and preserved following standard protocols. All field collection tag numbers or, when available, museum catalog numbers associated with specimens used in this study are listed in supplementary Table S1, Appendix.

Host Ecological Data and Phylogeny

Data on host ecology and life history were compiled based on personal observations and a thorough review of published literature (Table S1). Our taxonomy follows that of the EMBL Reptile Database (http://www.reptile-database.org/ last accessed February, 2017). For statistical analyses of microbial variation with respect to host phylogeny we used phylogenetic hypotheses of host relationships based on both molecular (Pyron *et al.* 2013) and morphological and molecular (Townsend *et al.* 2004; Conrad 2008) data.

Microbiome Sampling and Library Construction

All individuals were field collected via one of the following methods: 1) active searches in suitable habitat, 2) road surveys, 3) drift fence and pitfall trap arrays. Individuals capture via trapping were removed from traps within 24 hours. Gut microbial samples were collected via cloacal swabs following standard protocols for reptiles (Colston *et al.* 2015b) and were immediately frozen until extraction. Microbial DNA was extracted by a bead beating procedure using MoBio Power Soil Extraction kits (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's standard DNA extraction protocol with minor adjustments (Colston *et al.*

2015b). PCR was performed in multiplex on standardized DNA extractions and blank negative controls to amplify the V4 region of the 16S rRNA gene using the primers 16Sf and 16Sr (Kozich *et al.* 2013). PCR libraries were then sent to the University of Mississippi Medical Center Molecular and Genomics Core sequencing facility for sequencing on an Illumina MiSeq.

Analysis of Bacterial Inventories

Microbial sequences were processed and analyzed using mothur version 1.38.1 (Schloss *et al.* 2009). Following denoising and barcode removal sequences were aligned using the SILVA reference alignment (Pruesse *et al.* 2007; Quast *et al.* 2013). Sequences differing by only one nucleotide were grouped together and we checked for chimeric sequences using the chimera.slayer function in mothur. Chimeric sequences and any non-bacterial 16S sequences were removed. We classified the remaining reads into operational taxonomic units (OTUs) based on 97% sequence similarity using the SILVA reference database (Konstantinidis & Tiedje 2005; Quast *et al.* 2013).

The distribution of OTUs in each sample was used to compare several aspects of gut bacterial community diversity and structure. Because samples varied in the number of final valid sequences obtained, all analyses were performed on a randomly sampled subset of the total dataset for each sample, which corresponded to the number of sequences in the smallest sample (i.e. all samples were standardized to be equivalent to the sample with the lowest number of valid reads, in our case this was1,000) and this random subsampling was performed 1,000 times for each analysis (Kozich *et al.* 2013; Colston *et al.* 2015b). We determined alpha diversity within each sample using Schao and inverse Simpsons indices, and rarefaction and collection curves were used to visualize whether our procedures included enough sampling depth to assess this diversity. For beta diversity (comparisons of bacterial community structure between samples) we

calculated the Yue & Clayton theta similarity index (Yue & Clayton 2005) which accounts for proportional abundance of OTUs in a sample. Similarity between samples was visualized by non-metric multidimensional scaling (NMDS).

Additionally, we tested the spatial separation of samples based upon various ecological traits (e.g. diet, habitat, parity mode), host phylogeny and skull morphology through analysis of molecular variance (AMOVA). To identify specific OTUs that were driving observed differences we performed indicator analyses (Dufrêne & Legendre 1997). Indicator values (IV) range from 0–1 and only indicators with an IV>0.3 and a p-value<0.05 were considered good indicators (Fortunato *et al.* 2013). To further compare the relationship of bacterial community membership and structure among samples we constructed dendrograms based on Jaccard and theta distances (Zhang *et al.* 2014; Colston *et al.* 2015b). We then tested for significant clustering of community membership or structure in the resulting trees using parsimony (Zhang *et al.* 2014), and UniFrac unweighted and weighted distances (Lozupone & Knight 2005). We performed these analyses on the full dataset, a reduced dataset that contained squamate reptiles only, and on datasets that included lizards or snakes only.

RESULTS

Sequence Analyses

After alignment and removal of potential chimeras and non-bacterial sequences we recovered 10,426,180 valid sequence reads (average of 26,734 reads per individual), representing 48,046 unique sequences with a median length of 253 base pairs (bp). Our pooled dataset consisted of 390 individuals from five localities (Brazil, Ethiopia, Guyana, Mexico, United States) representing 22 families of squamates reptiles spanning the squamate tree of life (Figure 1, supplementary Table S1, Appendix), as well as turtles and crocodilians as reptilian outgroups.

Sequences were binned into 8,945 OTUs and rarefaction suggested that we recovered >95% of the diversity in all but six of our samples with 91%-94% of diversity recovered in those six samples (supplementary Table S2, Appendix). Approximately 3% of our sequences (275,208 reads) could only be classified as "unclassified Bacteria" using the SILVA taxonomy database (Figure 2).

Proteobacteria Firmicutes Bacteroidetes Bacteria_unclassified Actinobacteria Fusobacteria Other_Phyla
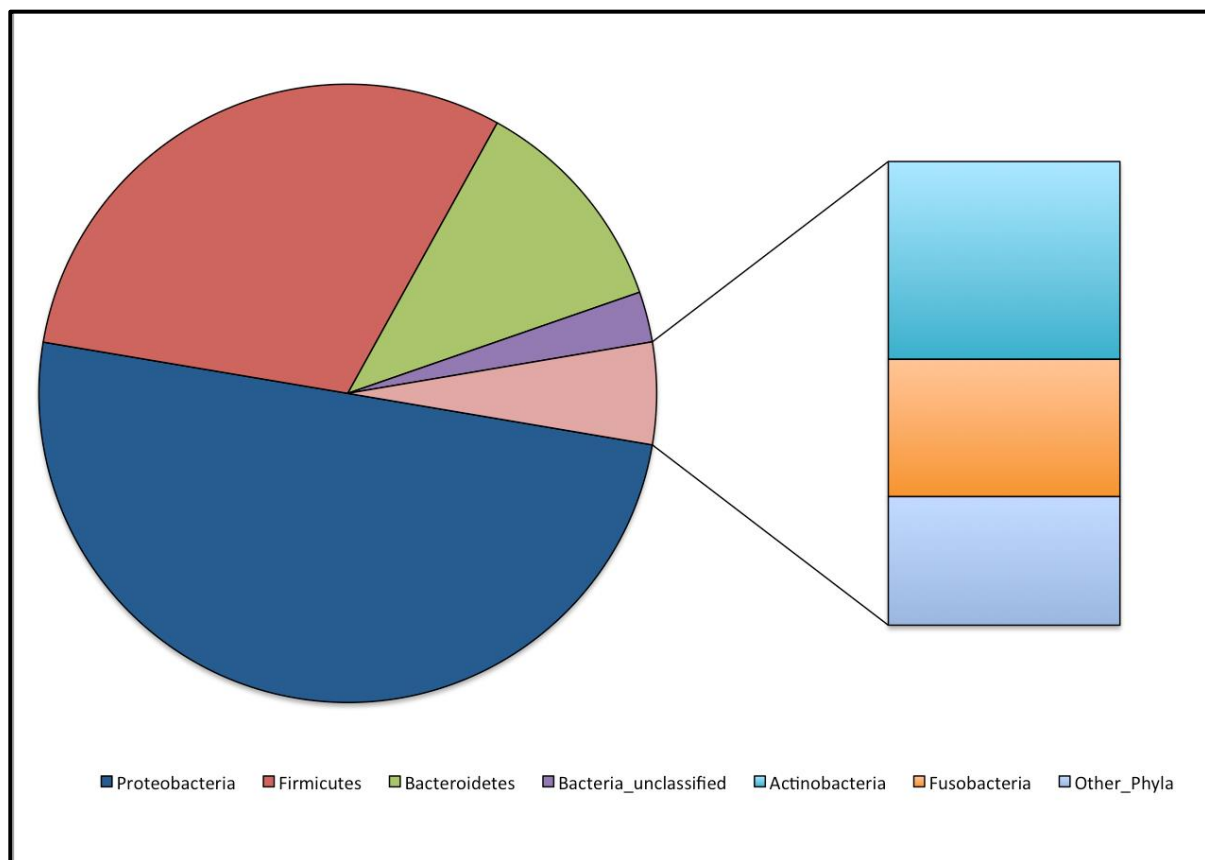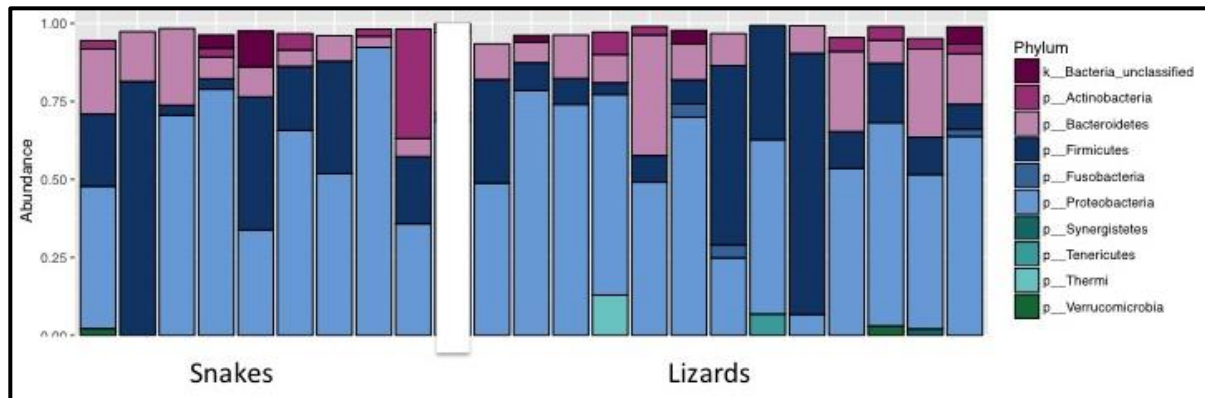
Figure 2. Percentage of bacterial phyla in pooled dataset of 390 reptile gut microbial samples. Above) Sequences were classified according to the SILVA database. Colors correspond to bacterial phyla listed below. Blow out is the percentage of bacterial phyla representing a combined total of approximately 5% of all pooled sequences. Below) Relative abundance of bacterial phyla in snakes (left) and lizards (right) with samples along the x axis binned by host family or subfamily. These sequences represent all bacterial phyla with >2% relative abundance

The core reptile microbiome is dominated by members of the Proteobacteria (50%), Firmicutes (30.3%) and Bacteroidetes (11.7%) (Figure 2). At a finer level, Gammaproteobacteria (38.5% of all sequences) were the most abundant class, and 55.3% of those sequences classified the family Enterobacteriaceae. *Bacillus* (Firmicutes) was by far the most dominant genus, accounting for 15% of all sequences. Microbiome samples did not group by reptilian order (Crocodilia–Squamata–Testudines; AMOVA: df=2 F_s=1.091 p=0.291). However, significant differences in the microbiomes of Order Squamata were detected between lizards and snakes (Lacertilia–Serpentes; AMOVA: df=1 F_s=2.375 p=0.007), and indicator analysis identified 27 OTUs that drove these differences (Table 1; Figure 3). Gut bacterial community composition significantly varied by host geographic locality at the country scale (AMOVA: df=4 F_s =7.879 p= <0.001; Table 2; Figure 4).

Our data were highly dimensional, and NMDS stress levels approached 0.2 in three dimensions (Figure 4). Parsimony tests confirmed significant differences in microbiome composition between lizards and snakes (p <0.001), and UniFrac analyses detected significant

differences in both microbiome membership (UWscore=0.993 UWsig=<0.001) and community

structure (Wscore=0.628 Wsig=<0.001) between lizards and snakes (Bonferoni corrected

alpha=0.008). Therefore all remaining analyses were performed on lizard and snake datasets

separately.

Table 1. Bacterial OTUs that show significant differences in their distribution across the lizard and snake microbiome. OTU number, classification, whether OTU was elevated (more abundant) in lizards or snakes, indicator value (IV) and p-value of OTUs found to be significant in indicator analyses.

OTU	Classification	Elevated	IV	*p*
Otu00006	Alcaligenaceae	snakes	0.745	<0.001
Otu00004	*Morganella*	snakes	0.675	0.038
Otu00037	*Comamonas*	snakes	0.659	0.014
Otu00005	Gammaproteobacteria_unclassified	snakes	0.647	0.025
Otu00032	*Paracoccus*	snakes	0.614	<0.001
Otu00020	Rhodobacteraceae	snakes	0.576	<0.001
Otu00011	Neisseriaceae	snakes	0.563	0.016
Otu00008	Gammaproteobacteria_unclassified	snakes	0.559	0.028
Otu00085	Burkholderiales_unclassified	lizards	0.554	0.042
Otu00018	Brucellaceae	snakes	0.537	0.017
Otu00220	*Janibacter*	lizards	0.437	0.035
Otu00074	*Corynebacterium*	lizards	0.414	0.005
Otu00244	*Herbaspirillum*	lizards	0.400	0.033
Otu00092	*Kocuria*	lizards	0.395	0.002
Otu00054	*Fusobacterium*	snakes	0.387	0.03
Otu00133	*Alistipes*	lizards	0.385	0.003
Otu00261	Lachnospiraceae	lizards	0.379	<0.001
Otu00057	*Aeromonas*	lizards	0.378	0.019
Otu00175	*Bacteroides*	lizards	0.375	<0.001
Otu00039	*Vagococcus*	snakes	0.363	0.049
Otu00088	*Akkermansia*	lizards	0.361	0.03
Otu00031	*Mycoplasma*	snakes	0.356	0.042
Otu00084	*Alistipes*	snakes	0.342	0.009
Otu00144	*Tsukamurella*	snakes	0.327	<0.001
Otu00235	Porphyromonadaceae	lizards	0.310	0.002
Otu00443	*Methylobacterium*	lizards	0.306	0.007
Otu00409	*Cupriavidus*	lizards	0.301	0.033
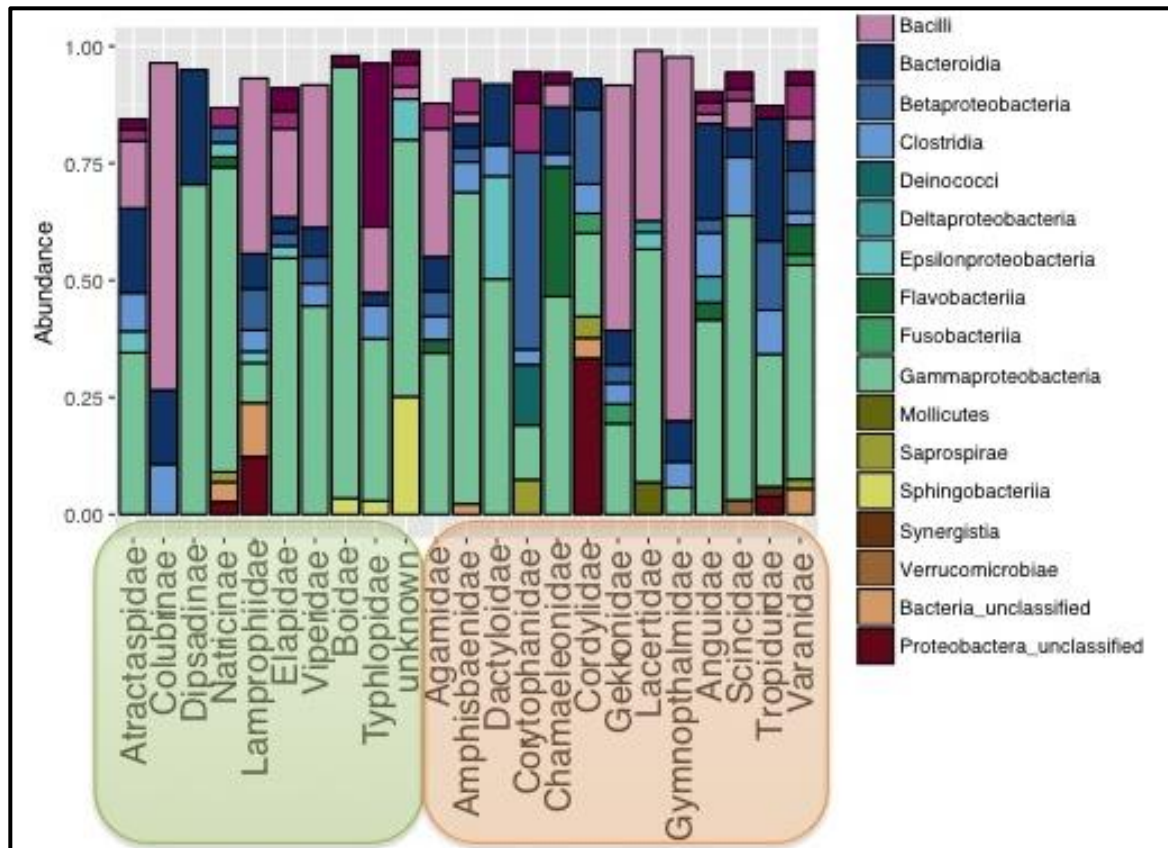
Figure 3. Relative abundance of bacterial classes present in Squamate reptile gut microbial samples. Samples are displayed binned to host family or subfamily with lizard taxa shaded in orange and snake taxa are shaded in green. These sequences represent all bacterial classes with >2% relative abundance.

Table 2. Gut bacterial community is structured significantly by geographic location of the host. AMOVA results for tests we made using the full dataset of all squamate reptile gut bacterial samples. All comparisons were significant at $p<0.005$ after Bonferoni correction.

Source of variation	F_s	p
Brazil-Ethiopia-Guyana-Mexico-USA	7.8791	<0.0001
Brazil-Ethiopia	4.13041	<0.0001
Brazil-Guyana	20.4085	<0.0001
Brazil-Mexico	2.81203	<0.0001
Brazil-USA	5.82413	<0.0001
Ethiopia-Guyana	21.3385	<0.0001
Ethiopia-Mexico	3.79583	<0.0001
Ethiopia-USA	4.46649	<0.0001
Guyana-Mexico	17.9237	<0.0001
Guyana-USA	11.9194	<0.0001
Mexico-USA	2.94854	<0.0001
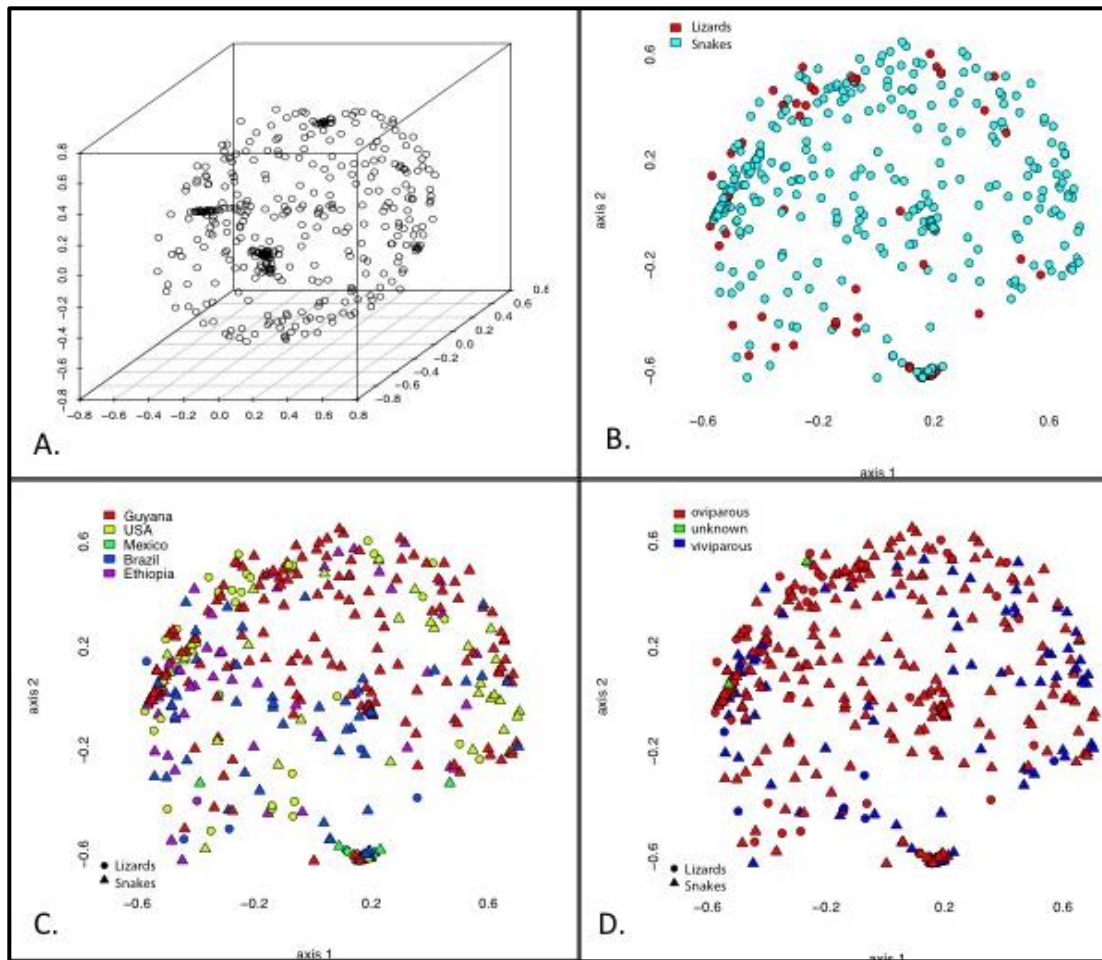
Figure 4. Nonmetric multidimensional scaling plots of bacterial community structure based on Yue & Clayton theta (thetayc) similarities of bacterial communities. Plots are in 3 dimensions (stress=.25). A) Plot of lizards and snakes in all three dimensions B) lizards and snakes colored by suborder in two dimensions C) Plot of lizards and snakes coded by suborder and country locality in two dimensions C) Plot of lizards and snakes coded by suborder and parity mode in two dimensions.

Lizards

Differences in lizard gut microbiome composition and community structure were examined

based on ecological correlates (i.e. dietary preferences correlated with morphology) related to

deep evolutionary hypotheses based on morphology (Vitt & Pianka 2005). There were no

significant differences in lizard gut microbiome membership regardless of ecological or

phylogenetic grouping, as determined from Parsimony based on Jaccard dissimilarity or UniFrac

analysis of unweighted distances. Microbiome structure did not differ phylogenetically (AMOVA: df=1 F_s=1.120 p=0.298) when lizards were grouped based on traditional phylogenetic hypotheses based on morphology and limited molecular data (Townsend *et al.* 2004). The structure of the lizard microbiome did differ based on parity mode (AMOVA: df=1 F_s=2.058 p=0.019), diet breadth (AMOVA: df=2 F_s=3.228 p=<0.001), foraging mode (AMOVA: df=2 Fs:2.479 p=0.008), habitat (AMOVA: df=4, F_s=2.086 p=0.0001) and molecular phylogeny (Pyron *et al.* 2013) (AMOVA df=12 F_s=1.720 p=<0.001). Specifically, gut bacterial community structure varied significantly between agamids and tropodurids (AMOVA: df=1 F_s=7.603 p=<0.001), and gekkonids and tropodurids (AMOVA: df=1 F_s=6.580 p=0.002) but not between other families examined.

Snakes

The snake microbiome varied with known ecological correlates (i.e. dietary preferences correlated with skull morphology) related to deep evolutionary hypotheses based on morphology (Colston *et al.* 2010). Microbiome composition and structure varied significantly between scleroglossan and alethinophidian snakes (parsimony: p=<0.001, UniFrac: UWscore 0.997 UWsig<0.001, Wscore0.825 Wsig<0.001, AMOVA: df=1 F_s=3.7394 p<0.001; Bonferoni corrected alpha=0.008), and the composition of the snake microbiome differed based on host activity, diet, parity, foraging mode, habitat and in some cases phylogeny (UW Unifrac Table 3).

Additionally, snake gut bacterial community structure differed significantly based on parity mode (AMOVA: df=2, F_s=3.090 p=<0.001), diet breadth (AMOVA df=3 F_s=2.767 p=<0.001), foraging mode (AMOVA: df=3 F_s=2.520 p=<0.001), habitat (AMOVA: df=7, F_s=3.061 p=<0.001), activity (e.g. diurnal, nocturnal etc. AMOVA: df=3 F_s=2.460 p=<0.001) and molecular phylogeny (Pyron *et al.* 2013) (AMOVA: df=9 F_s=5.236 p=<0.001). Specifically,

snake microbiome community structure varied between all families and subfamily comparisons

with the exception of atractaspids (all comparisons), boids and natricines, and colubrines and

dipsadinaes.

Table 3. Summary of unweighted UniFrac tests for significant phylogenetic clustering of gut bacterial community membership in snakes. Bonferoni corrected UWsig=<0.001 is significant.

Group Comparison	UWscore	UWsig
diurnal-nocturnal	0.986	<0.001
euryphagous-specialist	0.985	<0.001
euryphagous-stenophagous	0.987	<0.001
specialist-stenophagous	0.982	0.014
oviparous-viviparous	0.991	<0.001
active-ambush	0.991	<0.001
aquatic-arboreal	0.988	0.018
aquatic-fossorial	0.996	<0.001
arboreal-fossorial	0.991	0.026
aquatic-semi-aquatic	0.982	0.105
arboreal-semi-aquatic	0.976	0.272
fossorial-semi-aquatic	0.995	0.027
aquatic-semi-arboreal	0.986	0.008
arboreal-semi-arboreal	0.982	0.200
fossorial-semi-arboreal	0.990	0.001
semi-aquatic-semi-arboreal	0.982	0.130
aquatic-semi-fossorial	0.995	0.389
arboreal-semi-fossorial	0.973	0.788
fossorial-semi-fossorial	0.993	0.414
semi-aquatic-semi-fossorial	0.979	0.778
semi-arboreal-semi-fossorial	0.989	0.644
aquatic-terrestrial	0.993	<0.001
arboreal-terrestrial	0.991	0.020
fossorial-terrestrial	0.991	<0.001
semi-aquatic-terrestrial	0.991	0.044
semi-arboreal-terrestrial	0.986	0.010
semi-fossorial-terrestrial	0.995	0.467
Typhlopidae-atractaspidae	0.993	0.664
Typhlopidae-boidae	0.996	0.212
atractaspidae-boidae	0.992	0.926
Typhlopidae-colubrinae	0.991	0.005
atractaspidae-colubrinae	0.997	0.647

boidae-colubrinae	0.991	0.036
Typhlopidae-dipsadinae	0.994	<0.001
atractaspidae-dipsadinae	0.998	0.813
boidae-dipsadinae	0.994	0.004
colubrinae-dipsadinae	0.985	<0.001
Typhlopidae-elapidae	0.992	0.014
atractaspidae-elapidae	0.995	0.780
boidae-elapidae	0.995	0.231
colubrinae-elapidae	0.993	0.001
dipsadinae-elapidae	0.992	0.001
Typhlopidae-lamprophiidae	0.991	0.182
atractaspidae-lamprophiidae	0.992	0.973
boidae-lamprophiidae	0.995	0.449
colubrinae-lamprophiidae	0.993	0.033
dipsadinae-lamprophiidae	0.995	0.004
elapidae-lamprophiidae	0.978	0.331
Typhlopidae-natricinae	0.999	0.039
atractaspidae-natricinae	0.998	0.860
boidae-natricinae	0.987	0.263
colubrinae-natricinae	0.986	0.003
dipsadinae-natricinae	0.990	<0.001
elapidae-natricinae	0.996	0.031
lamprophiidae-natricinae	0.996	0.113
Typhlopidae-viperidae	0.993	0.001
atractaspidae-viperidae	0.997	0.428
boidae-viperidae	0.989	0.032
colubrinae-viperidae	0.988	0.001
dipsadinae-viperidae	0.990	<0.001
elapidae-viperidae	0.993	<0.001
lamprophiidae-viperidae	0.995	0.008
natricinae-viperidae	0.988	0.001

DISCUSSION

Wild squamate reptiles possess a core gut microbiome that is dominated by
Proteobacteria and Firmicutes and this microbiome differs between lizards and snakes (Figure 2;
Figure 3). While earlier and more limited studies on reptile gut microbiomes suggested that the
core microbial community was similar to that of mammals (Costello *et al.* 2010; Hong *et al.*

2011) our findings here emphasize the distinctness of the reptile gut microbiome and support the hypothesis that the reptile gut microbiome is more similar to that of birds (Hird *et al.* 2014, 2015b; Colston *et al.* 2015b). Findings from prior studies are limited by the effects of captivity (Costello *et al.* 2010; Kohl *et al.* 2016) and sampling design (Hong *et al.* 2011). Captivity has been shown to alter the gut microbial composition in lizards (Kohl *et al.* 2016) and while the authors of that study did not report the dominant phyla in wild collected adults, Bacteroidetes were significantly enriched in captive individuals when compared to their wild collected mothers (which were enriched with Firmicutes).

Little is known of how reptiles acquire their gut microbiota (Colston & Jackson 2016), but the gut microbiome differs in both community membership and structure from that of potential prey or the environment (Costello *et al.* 2010; Kohl *et al.* 2016). Lizards might acquire their endogenous microbiome via coprophagy, vertical transmission from mothers to offspring, environmental sources, or through close associations with conspecifics (Colston 2017). Our findings highlight that parity mode plays a significant role in gut microbiome structure in both lizards and snakes, so that mode of reproduction likely influences gut bacterial composition in all squamates. Future studies should specifically test how microbial communities are transferred from mothers to offspring, comparing live-bearing to egg laying groups.

Changes in foraging mode have ecological consequences and impact diversification in lizards (Huey & Pianka 1981; Cooper 1995; Costa *et al.* 2008) and the same concept can be applied to snakes. Active foraging reptiles use chemical cues to detect and discriminate prey items, are exposed to a wider array of predatory pressures and generally have a wider dietary breadth than sedentary foragers. These differences in foraging mode have a significant

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relationship with gut microbiome structure, although this is likely correlated with several factors such as diet breadth and habitat preferences.

While the composition of the reptile gut microbiome was significantly structured with respect to host phylogeny (i.e. family or sub-family) in many cases, this was not the case for all comparisons. Similarity in gut bacterial composition between different clades likely arises from convergence on similar ecologies (e.g. fossoriality) or the degree of dietary specialization found in others (e.g. dispsadine snakes). That said, the lizard microbiome could be distinguished from that of snakes, indicating an influence of evolutionary history on gut microbial community structure. Within snakes, our finding that skull morphology (i.e. scolecophidian vs alethinophidian snakes) corresponds to significant changes in gut bacterial community structure reveals that host biology (which in turn constrains ecology) also has an impact on the microbiome.

Traditional hypotheses of squamate phylogenetic relationships based on mitochondrial DNA and morphology supported an early division within squamates, separating the clade into two monophyletic groups, Iguania (including chameleons, agamids, iguanids), and Scleroglossa (including skinks, geckos, snakes, and amphisbaenians) (Caldwell 1999; Townsend *et al.* 2004b; Conrad 2008). Ecological studies supported the hypotheses that this early division was coupled with a stark contrast in ecological trajectories, and dietary niche preferences were given as evidence supporting this hypothesis (Vitt & Pianka 2005; Colston *et al.* 2010). Recent molecular phylogenetic analyses with improved taxon sampling based on both mitochondrial and nuclear genes, and sequence data derived from cDNA libraries of venom glands, have shown this hypothesis to be incorrect, likely due to convergence. The hypothesis that Iguania, anguimorphs and snakes form a monophyletic clade (Toxicofera) and share the derived trait of possessing

genes for venom secreting glands is now well supported (Vidal & Hedges 2005; Fry *et al.* 2006; Pyron *et al.* 2013). This interpretation has greatly increased our understanding of Toxicofera ("advanced squamates") and the diversity of niches they exploit, as this group includes herbivorous, carnivorous, and omnivorous species that occupy nearly all niches exploited by other squamates and have undergone accelerated rates of speciation (Pyron & Burbrink 2014; Feldman *et al.* 2016). Our data show that gut microbiome structure varies significantly with respect to traits associated with accelerated speciation rates in squamates and generally reflects host phylogeny. Future studies should incorporate functional metagenomics to investigate whether ecologically similar, but phylogenetically distant, species have converged on microbiome function as has been observed in mammals (Muegge *et al.* 2011).

Our findings highlight the importance of the gut microbiome in both host ecology and diversification, as changes in gut bacterial community structure are significantly associated with traits known to effect diversification rates in reptiles (Cooper 1995; Pyron & Burbrink 2014). With this unprecedented dataset we have shown that the vertebrate gut microbiome is not uniform in its composition across the squamate tree of life, despite carrying out many of the same necessary processes (e.g. nutrient acquisition, immunological function) for the host. We suggest that these differences are correlated with host physiology, specifically endothermy and reproduction. Placental mammals acquire much of their core microbiota via maternal transmission during birth (Ley *et al.* 2008a) and while other vertebrates such as fish and birds seem to acquire their gut microbiota from the environment (Hird *et al.* 2014; Ghanbari *et al.* 2015), this does not seem to be the case in reptiles and warrants further investigation.

Acknowledgements

CHAPTER IV

INVESTIGATING THE INFLUENCE OF THE MICROBIOME ON HOST COMMUNITY ASSEMBLY USING SNAKES AND THEIR GUT BACTERIA

Original Article (to be submitted to *The ISME Journal*)

Title: Investigating the influence of the microbiome on host community assembly using snakes and their gut bacteria.

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INTRODUCTION

Biogeographical, evolutionary, and ecological processes interact to determine the distribution of species and ultimately the assembly of communities. Three main hypotheses exist to explain this interaction: 1) That niche-related processes govern community assembly and local rules (e.g. competitive exclusion) dictate a species' ability to persist; 2) That species are ecologically equivalent, causing communities to assemble according to random speciation/extinction events; or 3) That starting conditions and historical patterns of speciation and extinction influence assembly more than local processes (McPeek 2008; Cavender-Bares *et al.* 2009). However, most studies of community assembly have focused on single guilds or trophic levels, with limited ability to detect broad assembly patterns or interaction effects between trophic levels. Whether or not interacting trophic levels are governed by the same assembly rules is a question that has been rarely explored.

Phylogenetic data provide the necessary information to quantify and test the processes that have shaped the evolutionary and ecological history of species, species assemblages, and communities (Webb *et al.* 2002a; Kraft *et al.* 2007) Organisms living together in a local community are representatives of a larger regional pool and possess the necessary physiological characteristics to occupy their environment and coexist with other species in their community (Cavender-Bares *et al.* 2009). Coexistence of community members is usually considered within the same trophic level or guild, but animals also harbor beneficial microorganisms that aid in

physiological processes (Garcia *et al.* 2014). In vertebrates, the vast majority of these microorganisms are found in the gut and collectively the genomes of these microorganisms may contain 10-100 times as many genes as the host's genome (Berg 1996; Sender *et al.* 2016). These microbes (collectively, the "microbiome") aid in the host's nutrient acquisition and immune response, and can influence host behavior, development, reproduction and overall health (Fraune & Bosch 2010; Colombo *et al.* 2015).

While habitat filtering has often been shown to be the driving force influencing assembly (particularly in sedentary organisms), we now know that this is due to ecological traits dictating community assembly processes (Cavender-Bares *et al.* 2009). However, to what extent do endogenous microorganisms influence their host communities? Soil bacteria influence both the abundance and diversity of plant communities, even driving the operation of terrestrial ecosystems (van der Heijden *et al.* 2008), but this question has been little explored in vertebrate animal systems. However, the apparent relationship between host phylogeny (or genotype) and microbial community composition has led to much discussion of co-evolution of microbial communities and their multicellular hosts (Ochman *et al.* 2010; Anderson *et al.* 2012; Phillips *et al.* 2012b; Moeller & Ochman 2014).

Both host diet and phylogeny have been shown to be important predictors of host endogenous (gut) microbial community composition (Colston *et al.* in prep; Ley *et al.* 2008, 2009; Sullam *et al.* 2012). The ability of selection to act upon factors influencing gut microbiota community structure is thought to be temporally constrained such that phylogenetic divergence within host samples are the broadest time scale and lifespan changes within an individual host such as age, growth and pregnancy are the shallowest temporal scale that can affect the community structure of gut microbiota (Phillips *et al.* 2012).

It has been suggested that within reptiles the endogenous microbiome can be considered an ecological trait that may influence host speciation (Colston & Jackson 2016; Colston *et al* in prep) but this has not been tested within a community assembly framework. Do shifts in host ecology correlate with shifts in endogenous microbial communities? Do microbial communities influence vertebrate host diversity by providing or limiting exploitable niche space? These questions could be addressed with a well-sampled system of vertebrate host and endogenous microbial communities.

Reptiles, and snakes in particular, are good candidates for investigating the influence of the microbiome on host community assembly as these organisms do not have the dispersal capabilities of other groups (e.g. birds), are easily sampled for their gut microbial communities (Colston *et al.* 2015a) and in most cases their populations have not been affected by fragmentation to the extent of other taxa (e.g. mammals) (Debinski & Holt 2000; Neto *et al.* 2012). Additionally, snake community diversity varies considerably in different biomes, and individual snake species differ greatly in their period of activity, habitat use, and diet specialization (Greene 1983, 1997). We assembled a dataset of three New World snake communities, including data on their ecologies and life history traits, and sampled representative species for their endogenous microbiomes. Using these data and molecular phylogenies of the hosts we test whether the endogenous microbiome of snakes is indicative of snake ecology or phylogeny and explore its influence on host community assembly.

MATERIALS AND METHODS

Study Systems

Through field surveys conducted from 2011-2014 we collected species occurrence and endogenous microbiome samples from three New World snake communities (one temperate,

Apalachicola National Forest, Florida, USA; one sub-tropical, Calakmul Biosphere Reserve, Mexico; and one tropical, Reserva Biológica Guaribas, Brazil; Table 1). These communities differ in latitude and species composition, and in the biogeographic histories of their assemblages. The temperate snake community was largely comprised of species with major radiations in North America; the tropical snake community was comprised of species with South American origins; whereas the subtropical community in Central America was a mix of species with North and South American affinities. Representatives of all snake species we recorded in each community were sampled for their endogenous microbiomes via cloacal swabs following standard protocols for sampling reptile microbiomes (Colston *et al.* 2015a). Snakes were either released after sampling, or euthanized and preserved for deposition in the natural history collection at Universidade Federal da Paraíba, João Pessoa, Brazil. All field collection tag numbers or, when available, museum catalog numbers associated with specimens used in this study are listed in supplementary Table S1. Sampling was conducted in accordance with IACUC protocols approved by the Committee for Animal Care and Use at the University of Mississippi (#13-02 & #13-04).

Table 1. Summary of snake host species used in this study. Samples are from three snake communities in Brazil, Mexico and the United States. Number of individuals used (#) and type of community: tropical (T), sub-tropical (ST), or temperate (Te) are indicated.

Brazil			Mexico			United States		
Host Species	#	Type	Host Species	#	Type	Host Species	#	Type
Amerotyphlops brongersmianus	5	T	*Boa constrictor*	1	ST	*Agkistrodon contortrix*	2	Te
Amerotyphlops paucisquamus	5	T	*Bothrops asper*	5	ST	*Agkistrodon piscivorus*	3	Te
Boa constrictor	1	T	*Coniophanes imperalis*	5	ST	*Cemophora coccinaea*	1	Te
Bothrops leucurus	5	T	*Coniophanes schmidti*	3	ST	*Coluber constrictor*	3	Te
Bothrops lutzi	1	T	*Dipsas brevifacies*	2	ST	*Crotalus adamanteus*	1	Te
Bothrops neuweidi	1	T	*Drymarchon melanurus*	1	ST	*Diadophis punctatus*	1	Te
Chironius sp.	3	T	*Drymobius margaritiferus*	2	ST	*Farancia abacura*	1	Te
Chironius flavolineatus	3	T	*Ficimia publia*	1	ST	*Heterodon platyrhinos*	1	Te
Drymoluber dichrous	1	T	*Immantodes cenchoa*	1	ST	*Lampropeltis extenuata*	1	Te
Epicrates assisi	3	T	*Immantodes tennusissimus*	1	ST	*Lampropeltis getula*	3	Te
Erythrolamprus almadensis	1	T	*Lampropeltis triangulum*	2	ST	*Masticophis flagellum*	1	Te
Erythrolampus peocilogyrus	5	T	*Leptodeira frenata*	4	ST	*Micrurus fulvis*	1	Te
Erythrolampus taeniogaster	4	T	*Leptodeira septentrionalis*	3	ST	*Nerodia clarkii*	4	Te
Helicops angulatus	5	T	*Leptophis mexicanus*	1	ST	*Nerodia fasciata*	5	Te
Immantodes cenchoa	2	T	*Micrurus diastema*	2	ST	*Nerodia floridana*	1	Te
Leptophis ahaetulla	2	T	*Ninea diademata*	2	ST	*Nerodia taxispilota*	1	Te
Lygophis dilepis	2	T	*Ninea sebae*	2	ST	*Opheodrys aestivus*	1	Te
Micrurus sp.	2	T	*Oxybelis aeneus*	1	ST	*Pantherophis guttatus*	2	Te
Micrurus aff. ibiboboca	5	T	*Pseudelaphe flavirufa*	2	ST	*Pantherophis obsoletus*	5	Te
Micrurus ibiboboca	4	T	*Psuestes poecilonatus*	1	ST	*Pituophis melanoleucus*	1	Te
Micrurus lemniscatus	4	T	*Tropidodipsas fasciata*	1	ST	*Reginia rigida*	2	Te
Oxybelis aeneus	3	T	*Tropidodipsas sartorii*	2	ST	*Sistrurus milarius*	5	Te
Oxyrhopus petolarius	1	T	*Xenodon rabdocephalus*	4	ST	*Thamnophis sauritus*	4	Te
Oxyrhopus trigeminus	5	T				*Thamnophis sirtalis*	4	Te

Philodryas nattereri	3	T
Philodryas olfersii	4	T
Philodryas patagoniensis	5	T
Psomophis joberti	1	T
Psuedoboa nigra	4	T
Sibon nebulatus	2	T
Sibynomorphus mikanii	5	T
Taeniophallus affinus	1	T
Tantilla melanocephala	5	T
Thamnodynastes pallidus	1	T
Xenodon merremi	2	T

Ecological and Phylogenetic Information

Ecological and life history data of snake hosts was compiled based on thorough reviews of published literature or collected ourselves (supplementary table S1; Appendix). For host phylogenetic information we use a published squamate phylogeny (Pyron *et al.* 2013) and the taxonomy of the EMBL reptiles database (http://www.reptile-database.org/ last accessed February, 2017). Representative phylogenies of snake species were constructed using existing data (Pyron *et al.* 2013) in the R program ape (Paradis *et al.* 2004) with the drop.tip function to prune trees to only those species for which we had microbial sampling, or a closely related representative species if a particular species was not present in the phylogeny of Pyron *et al.* (2013).

Bacterial Community Analyses – Bacterial 16S rRNA sequence data for representative snake species were extracted from a preexisting dataset (Colston *et al.*in prep). Samples were chosen to maximize both read quality and depth per sample, and the number of host species used ranged from 1-5 individuals (Table 1). Sequences were classified into operational taxonomic units (OTUs) based on 97% sequence similarity using the RDP reference database (V14; Wang *et al.* 2007) in the program mothur version 1.38.1 (Schloss *et al.* 2009). For further information on bacterial inventory sequence generation and quality checking see Colston *et al.*(in prep).

Snake bacterial communities are known to be influenced by host phylogeny and ecology (Colston *et al.* in prep). However it is unknown at what scale these factors operate. Therefore we analyzed bacterial communities in multiple ways: First, we tested for effects of sampling locality and phylogeny on the entire pooled dataset from all three snake communities. Second, we analyzed each of the three snake communities individually to test the effect of host phylogeny

and various ecological traits (e.g. diet, activity, parity mode; supplementary table S3; Appendix) on both bacterial community composition and structure.

OTU distribution in each sample was used to compare several aspects of gut bacterial community diversity and structure. Because samples varied in the number of final valid sequences obtained, all analyses were performed on a randomly sampled subset of the total dataset for each sample, which corresponded to the number of sequences in the smallest sample (in our case this was 1,063 sequences, supplementary table S3; Appendix) and this random subsampling was performed 1,000 times for each analysis (Kozich *et al.* 2013; Colston *et al.* 2015a). Alpha diversity within each sample was calculated using Chao1 and inverse Simpsons indices, and rarefaction and collection curves were used to visualize whether our procedures included enough sampling depth to assess this diversity. This was performed on the full dataset and on each of the three snake community datasets and significance was assessed with ANOVA. For beta diversity measures within snake communities (comparisons of bacterial community structure between samples) we first pooled all individual samples by host species (with randomly subsampling based on the lowest number of reads for any one sample) and then calculated the theta similarity index (Yue & Clayton 2005) which accounts for proportional abundance of OTUs in a sample. Similarity between samples was visualized by non-metric multidimensional scaling (NMDS).

We tested whether snake gut microbiome structure varied significantly with regard to a number of ecological traits that are ether reflective of niche (e.g diet, habitat) or known to influence speciation (e.g. parity mode, foraging mode) in snakes (Morales-Castilla *et al.* 2011; Pyron & Burbrink 2014) and host phylogeny within each of our three snake communities through analysis of molecular variance (AMOVA). Indicator analyses (Dufrêne & Legendre

1997) were used to identify specific OTUs that were driving observed differences. Indicator values (IV) range from 0-1 and only indicators with a IV>0.3 and a p-value<0.05 were considered good indicators (Fortunato *et al.* 2013). To further compare the relationship of community membership and structure among samples we constructed dendrograms based on Jaccard and theta distances (Zhang *et al.* 2014). We then tested for significant clustering of community membership or structure in the resulting trees using UniFrac unweighted and weighted distances (Lozupone & Knight 2005). To compare phylogenetic structure of gut communities with that of host phylogeny we constructed UPGMA dendrograms based on our UniFrac distance matrices (UDM). These UDMs were constructed for each host species in the three snake communities and distances were calculated based on the amount of branch length that was unique to either of two environments (Lozupone & Knight 2005; Hird *et al.* 2015b).

RESULTS

Snake Communities

214 individuals from 82 snake species were sampled (Table 1, supplementary table S3; Appendix). Based on previous work and our own observations this sampling is representative of the majority, if not all, snake species which are known to occur in these communities (França *et al.* 2012; Colston *et al.* 2015b). The tropical snake community had the highest species richness (35 species) followed by the temperate and subtropical communities with 24 and 23 species respectively.

Gut Bacterial Community Sequence Analyses

Following alignment and removal of any chimeric and non-bacterial sequences the dataset was comprised of 5,691,320 valid reads (average of 26,595 reads per individual), representing 23,631 unique sequences with a median length of 253 base pairs (bp). Sequences were binned into 5,325

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OTUs and rarefaction suggested that this represented >95% of the diversity in all but four of our samples (with 94% of diversity recovered in those four samples supplementary table S4; Appendix). Approximately 3% of our sequences (143,494 reads) could only be classified as "unclassified Bacteria" using the RDP taxonomy database. Consistent with previous studies of reptile microbiomes the snake gut bacterial community was dominated by members of the Proteobacteria (51%), Firmicutes (30.3%) and Bacteroidetes (12%). Bacterial community richness differed significantly between the three snake communities when compared using Chao1 (but not inverse Simpsons index) and Tukey HPD post hoc test of significance revealed that differences were significant between all comparisons ($p=<0.05$) with the exception of Mexico and the USA ($p=0.66$; Figure 1; Table 2). AMOVA revealed that gut bacterial community composition varied significantly by host community/geographic locality although this data was highly dimensional when visualized by NMDS (AMOVA: df=2 F_s =3.08181 $p=$ <0.001; Figure 2)**.** Therefore all remaining analyses were performed on each host community separately.

Table 2. Results of one-way ANOVA of measures of bacterial diversity in snake gut microbiomes compared across the three snake host communities. Two indices of diversity were calculated for each host species and the sample means were used in analyses.

Index	Source	*df*	*SS*	*MS*	*F*	*p*
Inverse Simpson's	Between Snake Communities	2	36.1	18.07	0.987	0.377
	Residuals	80	1464.1	18.3		
S_{Chao1}	Between Snake Communities	2	561622	280811	9.494	<0.001
	Residuals	80	2366268	29578		
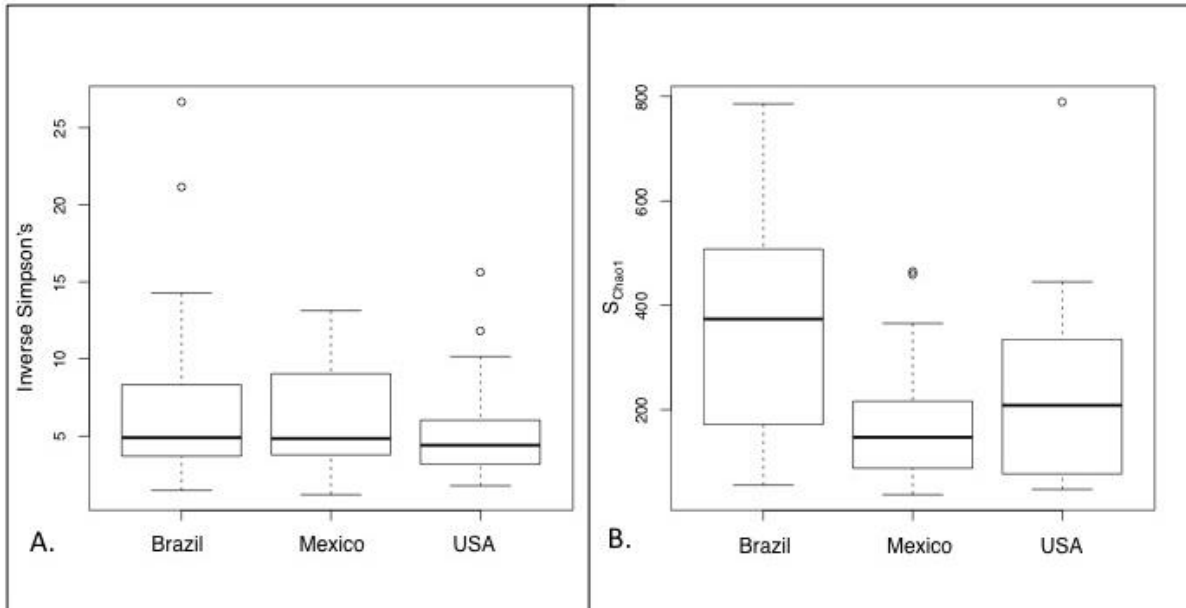
Figure 1. Diversity measures from the three snake host communities; gut bacterial samples from 36 snake species in Brazil, 24 snake species in Mexico and 23 snake species in the USA. A. Values of Inverse Simpson's index with maximum (upper bar), minimum (lower bar) and median (bold bar) and outlier (open circles) values indicated. B. Values of S_{Chao1} index with maximum (upper bar), minimum (lower bar) and median (bold bar) and outlier (open circles) values indicated.

Tropical Snake Community

The gut microbiome of the tropical snake community contained 3,911 bacterial OTUs dominated by members of Gammaproteobacteria, Bascilli, Bacteroidia, Betaproteobacteria, Alphaproteobacteria and Clostridia (Figure 3). Approximately 4% of the reads for our tropical dataset could only be classified to "Bacteria unclassified" which was the highest percentage of the three datasets. The dominant bacterial genera identified were *Bacillus*, *Bacteroides*, *Morganella,* and *Acinetobacter* representing 10%, 4%, 3% and 3%, of sequences respectively.

While dendrograms based on UDMs revealed that gut bacterial communities were not topologically congruent with host phylogeny (supplementary Figure S1a; Appendix) we detected significant differences in gut bacterial composition due to host taxonomic rank at the family-sub-

family level (AMOVA: df=5 F_s =2.72115 p= <0.001; Bonferoni corrected alpha=0.003 Figure
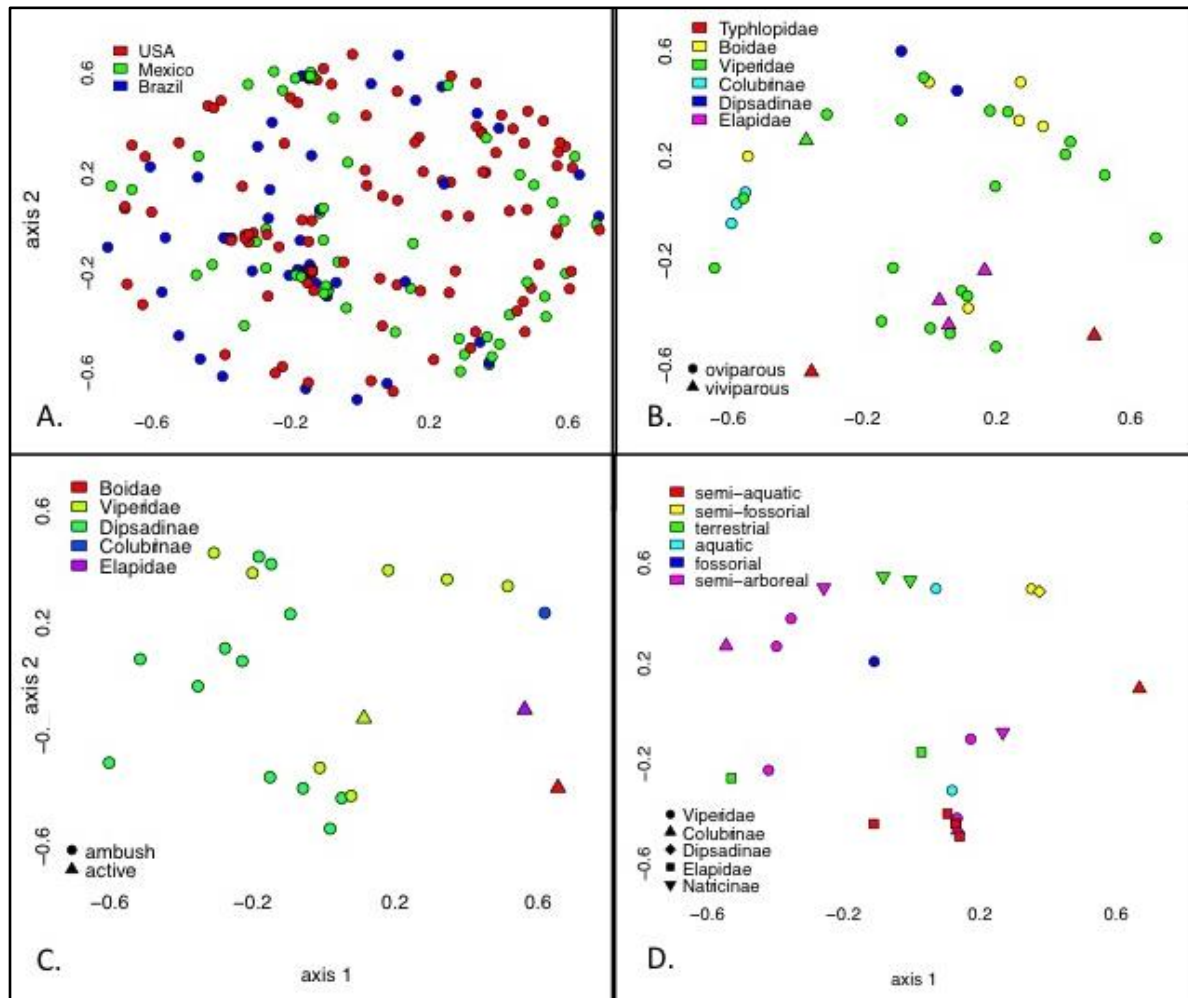
3).

Figure 2. Nonmetric multidimensional scaling plots of snake gut bacterial community structure based on Yue & Clayton theta (thetayc) similarities of bacterial communities. NMDS was plotted in three dimensions (stress<0.25) but only two dimensions are shown. A) All snake samples used in this study colored by country locality. B) Brazilian snake community samples coded by taxonomic rank and parity mode of hosts. C) Mexican snake community samples coded by taxonomic rank and foraging mode of hosts. D) USA snake community samples coded by taxonomic rank and habitat use of hosts.

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Figure 3. Relative abundance of gut bacterial subphyla in a tropical snake community. Host species are indicated on the x axis.
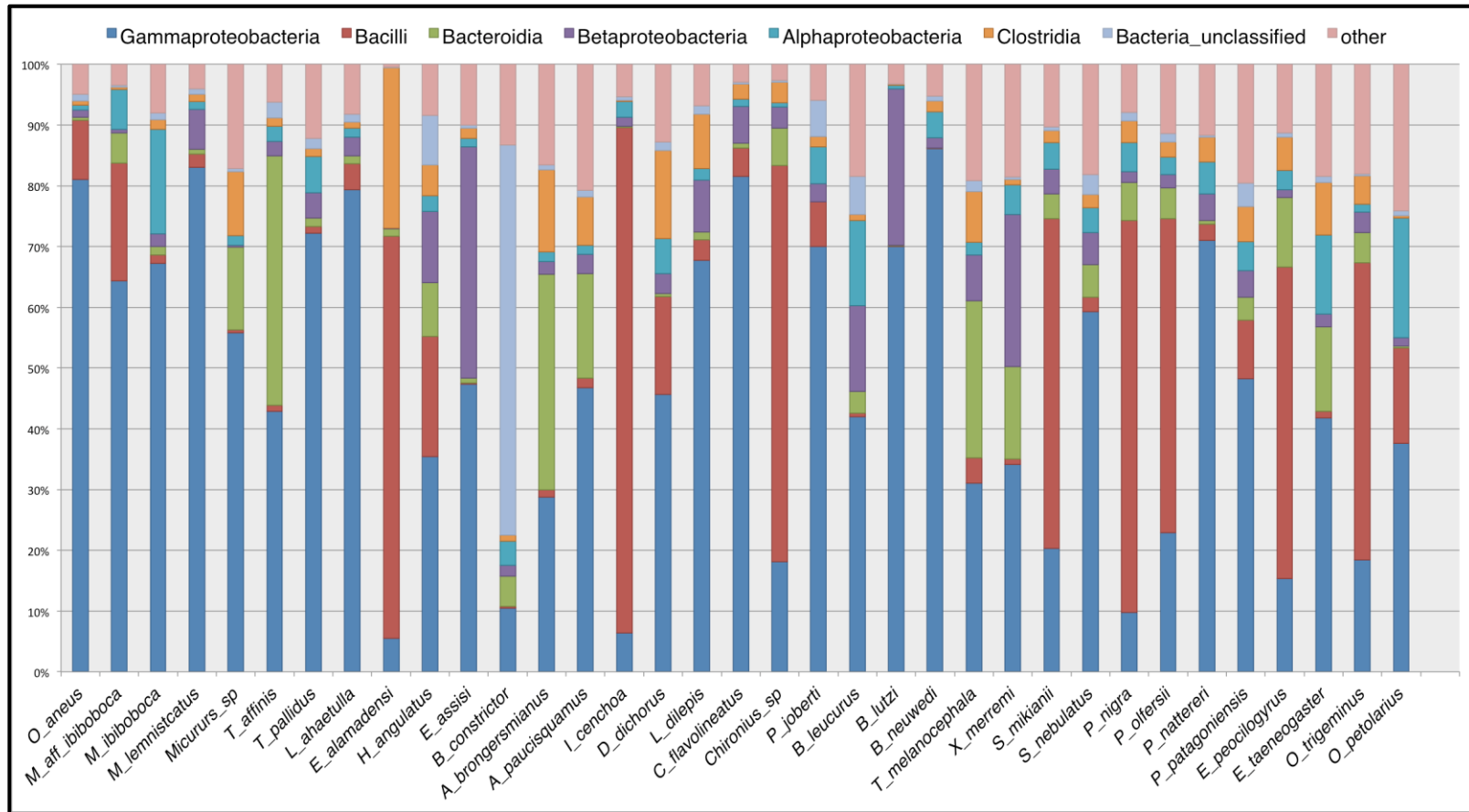
Pairwise comparisons between families reveled that this was driven by differences in gut community composition of dispsadine snakes and those of viperids and elapids (AMOVA: df=1 F_s =3.85026 p= <0.001). Indicator analyses revealed that tropical elapid and viperid snake bacterial communities were enriched with unique unclassified Proteobacteria compared to gut bacterial communities of dipsadines (IV>0.8, p=<0.001). Subsequent pooling of samples by family and dendrogram construction based on the resulting UDM showed some topological congruence with host family-level phylogeny (supplementary Figure S2; Appendix). Unifrac analyses based on weighted scores detected significant differences in gut bacterial community structure in all host taxonomic rank comparisons (Wscore=>0.9, Wsig=<.0001); but analyses based on unweighted scores were non-significant indicating that bacterial community membership did not vary according to host phylogeny.

Tropical snake gut bacterial community structure varied based on parity mode (Figure 2), host foraging mode and habitat but not according to diet breadth or host activity (Table 3). Indicator analyses revealed that tropical snake gut communities of oviparous species were enriched with the genus *Staphylococcus* and unclassified Enterobacteriaceae while gut communities of viviparous species were enriched with unclassified Gammaproteobacteria (IV>0.9, p=<0.0.5). Unclassified Gammaproteobacteria were enriched in the gut microbiomes of sit and wait/ambush predators (IV>0.9, p=<0.0.5) while active foragers had higher numbers of *Acinetobacter* although this result was not statistically significant (IV>0.9, p=0.08).

Sub-Tropical Snake Community

1,719 bacterial OTUs were recovered from the subtropical snake community, and these were dominated by members of the Gammaproteobacteria, Bascilli, Flavobacteria, Betaproteobacteria,

Bacteroidia, Alphaproteobacteria, and Clostridia with less than 1% of our reads classified as

"Bacteria unclassified" (Figure 4). The dominant bacterial genera were *Bacillus* (8% of reads),

Salmonella (5% of reads), *Providencia* (5% of reads), *Acinetobacter* (4% of reads), *Morganella*

(4% of reads), *Stenotrophomonas* (4% of reads), *Brucella* (3% of reads), and *Bacteroides* (3% of

reads.

Table 3. Gut bacterial community structure is significantly influenced by various ecological traits of hosts. AMOVA results for tests of significant variation in gut community structure for our three snake communities (*indicates significance).

Source	*df*	F_s	*p*
Brazil			
Foraging mode	1	3.205	0.002*
Diet breadth	2	0.941	0.523
Habitat	4	1.524	0.020*
Host activity	2	1.546	0.055*
Parity mode	1	3.255	0.001*
Mexico			
Foraging mode	1	1.744	0.047*
Diet breadth	2	0.923	0.562
Habitat	5	1.202	0.144
Host activity	1	1.297	0.193
Parity mode	1	1.229	0.229
USA			
Foraging mode	1	2.966	0.011*
Diet breadth	2	1.460	0.144
Habitat	5	1.702	0.025*
Host activity	1	0.608	0.760
Parity mode	1	1.171	0.255

Figure 4. Relative abundance of gut bacterial subphyla in a subtropical snake community. Host species are indicated on the x axis.
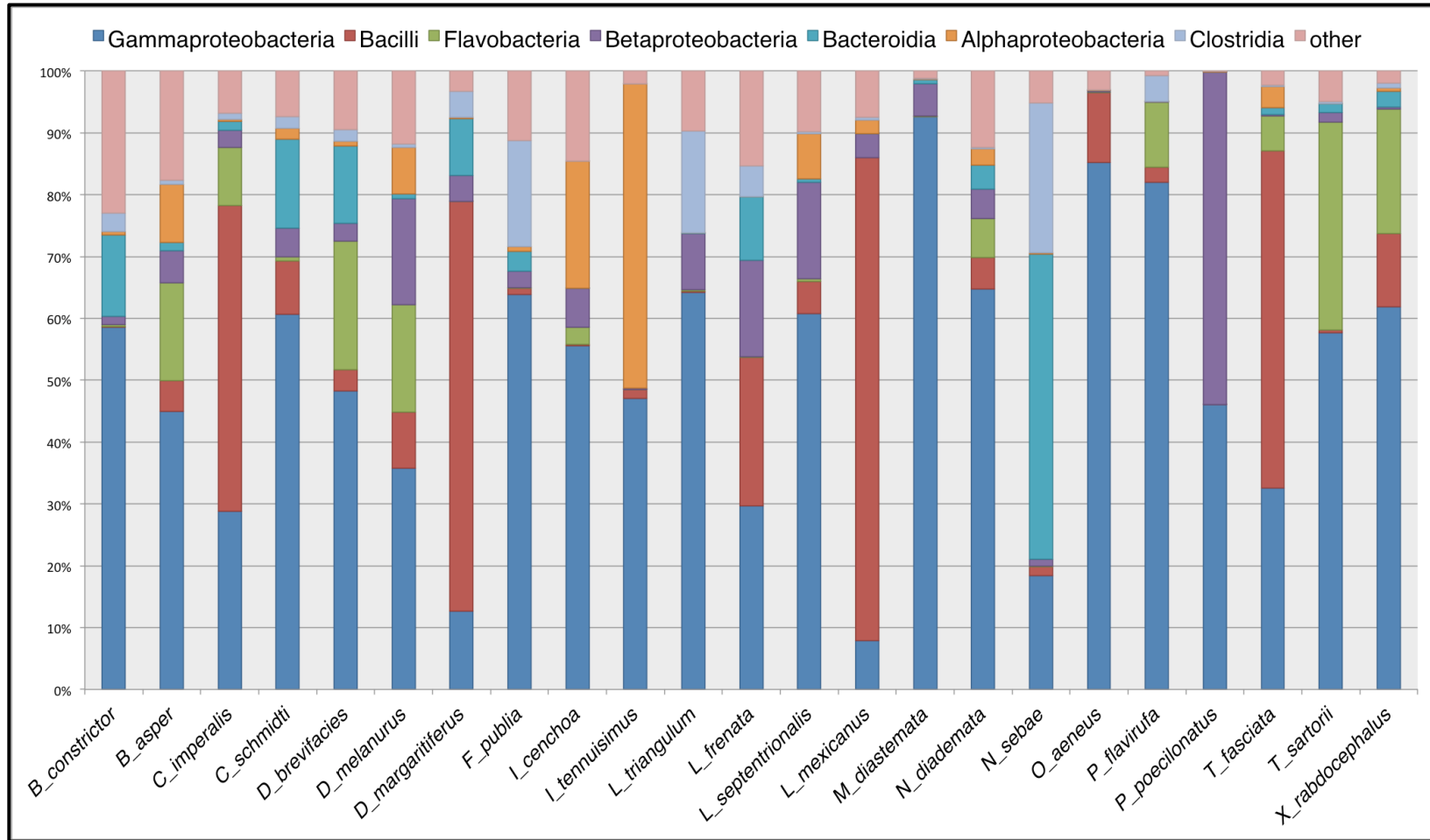
Dendrograms based on UDMs revealed that gut bacterial communities were not topologically congruent with host phylogeny (supplementary Figure S1b; Appendix). We detected significant differences in gut bacterial composition due to host taxonomic rank at the family sub-family level (AMOVA: df=4 F_s =1.69357 p= 0.002, Figure 3). However, pairwise comparisons between families were not significant and indicator analyses failed to detect any particular OTU that was significantly elevated in any single host family. As with the tropical snake community pooling samples by family and dendrogram construction based on the resulting UDM showed some topological congruence with host family-level phylogeny (supplementary Figure S2). Subtropical snake gut bacterial community structure varied based on foraging mode (Figure 2), but did not vary significantly by any other host traits examined (Table 3). Indicator analyses identified that sit and wait/ambush predators in the subtropical snake community had gut bacterial communities enriched with unclassified members of the genus *Flavobacterium* (IV>0.9, p=<0.02).

Temperate Snake Community

The gut bacterial composition of the temperate snake community was comprised of 1777 OTUs that were primarily members of the Gammaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Alphaproteobacteria, Colstridia and Flavobacteria (Figure 5). Approximately 2% of reads for our temperate snake community could only be classified to Bacteria with the RDP reference database. The only two identified genera that were represented in >3% of sequences were Bacillus (26% of reads) and *Acinetobacter* (5% of reads).

Figure 5. Relative abundance of gut bacterial subphyla in a temperate snake community. Host species are indicated on the x axis.
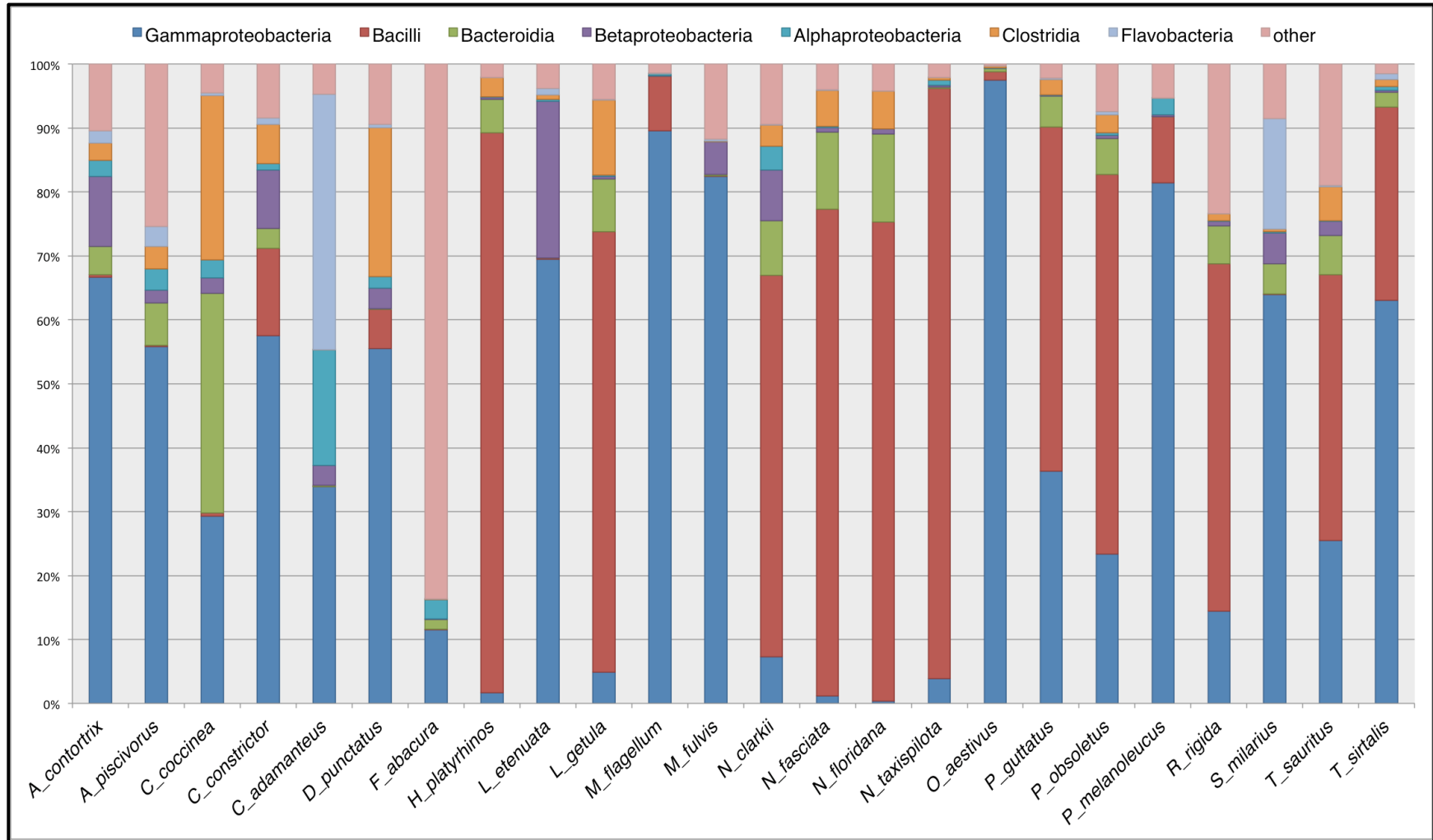
Dendrograms based on UDMs of the temperate host species gut bacterial communities were not topologically congruent with host phylogeny (supplementary Figure S1c; Appendix) but there were significant differences in gut bacterial composition based on host taxonomic rank at the family sub-family level (AMOVA: df=4 F_s = 2.35919 p= 0.002, Figure 3). Pairwise comparisons of taxonomic ranks were not significant with the exception of natricines and viperids (AMOVA: df=4 F_s = 10.3704 p= 0.003). Indicator analyses identified that natricine gut bacterial communities were enriched with the genera *Alistipes* and *Leptolinea* and unclassified Planctomycetaceae and Actinomycetales when compared to viperids. Temperate snake gut bacterial community structure varied based on foraging mode and habitat only (Figure 2, Table 3). Terrestrial viperids had gut bacterial communities enriched with unclassified Actinomycetales and this also drove differences detected in the gut bacterial communities of sit and wait vs. active foragers (IV>0.9, p=<0.02).

DISCUSSION

The microorganisms associated with vertebrates are essential for physiological function and efficient immune response and development, making them essential, but often unrecognized, components of biodiversity. Our results show that across three host communities, separated by thousands of kilometers and varying in their species composition, the gut microbiome of snakes is relatively conserved at the snake (host) family level. These findings support the notion that vertebrates are truly metagenomic organisms and that selection acts not only on the individual host but also on its resident microbiota (Zilber-Rosenberg & Rosenberg 2008).

Wild snakes possess a core microbiome that is largely comprised of members of the Gammaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Alphaproteobacteria, Colstridia and Flavobacteria. Our findings emphasize that the effect of geographic locality, on a broad

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scale, structures gut bacterial community membership and diversity. While our results may seem contradictory in that richness as estimated by Chao1 was not statistically significant between communities, this is likely a result of many rare taxa in our datasets. Richness estimators such as Simpson's index that rely on counts of singleton can be biased due to rare taxa, and Chao has been shown to be robust to these biases (He *et al.* 2013). Very few squamate reptiles have had their microbiomes characterized through NGS surveys (but see Colston *et al.* in prep) and our use of the RDP database as a conservative method of classifying our bacterial sequences resulted in many OTUs not classified beyond the family level.

Within each of the three host communities there was an effect of host phylogeny on gut bacterial community structure, but again this was limited to the taxonomic rank of host family or subfamily. While UniFrac analyses did detect an effect of phylogeny of gut bacterial community structure in all three host communities, dendrograms based on UDMs were not congruent with host phylogeny. We were limited in our abilities to test for significant clustering or over dispersion of bacterial communities with respect to host communities as it is necessary to have abundance data for the hosts (Webb *et al.* 2002). Additionally it has been suggested that it is difficult to detect the effects of competition in the assembly of bacterial communities due to broad taxonomic classification schemes indicative of 16S rRNA studies (Koeppel & Wu 2014); and we admit that more specific classification of bacterial taxa would be beneficial.

Across all three host communities foraging mode had a significant effect on gut bacterial community structure. More sedentary hosts presumably might undergo longer periods without meals as they wait for potential prey; and in snakes that are sit and wait predators consume relatively larger meals than more active hunters (Huey & Pianka 1981; Secor 2008). Gut community structure has been shown to change significantly during digestion (Costello *et al.*

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2010), with increases in particular bacterial phyla. Differential digestion times and frequency of meals could select for different gut microbiota in snakes. Habitat use had a significant effect on gut bacterial community structure in two of our snake communities. Habitat is likely correlated with both foraging mode and diet, but diet breadth did not have a significant effect on gut community structure. Diet breadth broadly effects the composition of squamate gut bacterial communities (Colston *et al.* in prep) but this effect may be lost when comparing individual hosts from the same local community. Additionally, little is known how snakes acquire their gut microbiota. It can be assumed that viviparous species acquire some microbes via maternal transmission during birth, but this has never been tested in snakes. Parity mode significantly influences the gut microbiome across Squamata, yet this effect was only detected in the tropical snake community. Here too it may be that local effects (i.e. sources of microbes) are simply stronger when comparing host species from a relatively small geographic area.

Many of the indicator taxa we identified in the gut microbiome of snakes are found in the intestinal or alimentary canals of other vertebrates, supporting the hypothesis that the endogenous microbiome is highly conserved (Ley *et al.* 2009). However, we also identified taxa that can be pathogenic in some animals as well as taxa that are often associated with soil or water samples. Without greater taxonomic resolution and functional metagenomic analyses it is unclear whether these indicator taxa are commensal parts of the ophidian microbiome, pathogens or simply transitory.

Our results indicate that the importance of phylogeny, geographic locality and ecology on structuring the gut microbiome of snakes varies depending on the community in question. Generally, the composition of the ophidian gut microbiome is foremost dependent on *who* (at the taxonomic level of family or subfamily) and *where* the snake is followed by *how* the snake lives.

The ophidian gut microbiome reflects ecological traits of the host species and therefore should be considered when investigating the influence of ecology on community assembly. There has been increased interest in making the collection of microbiome samples standard practice in ecological and evolutionary studies (Amato 2013; Hird *et al.* 2015) and we feel that characterizing the microbiome is an essential component to understanding an organisms natural history.

Acknowledgements

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APPENDIX
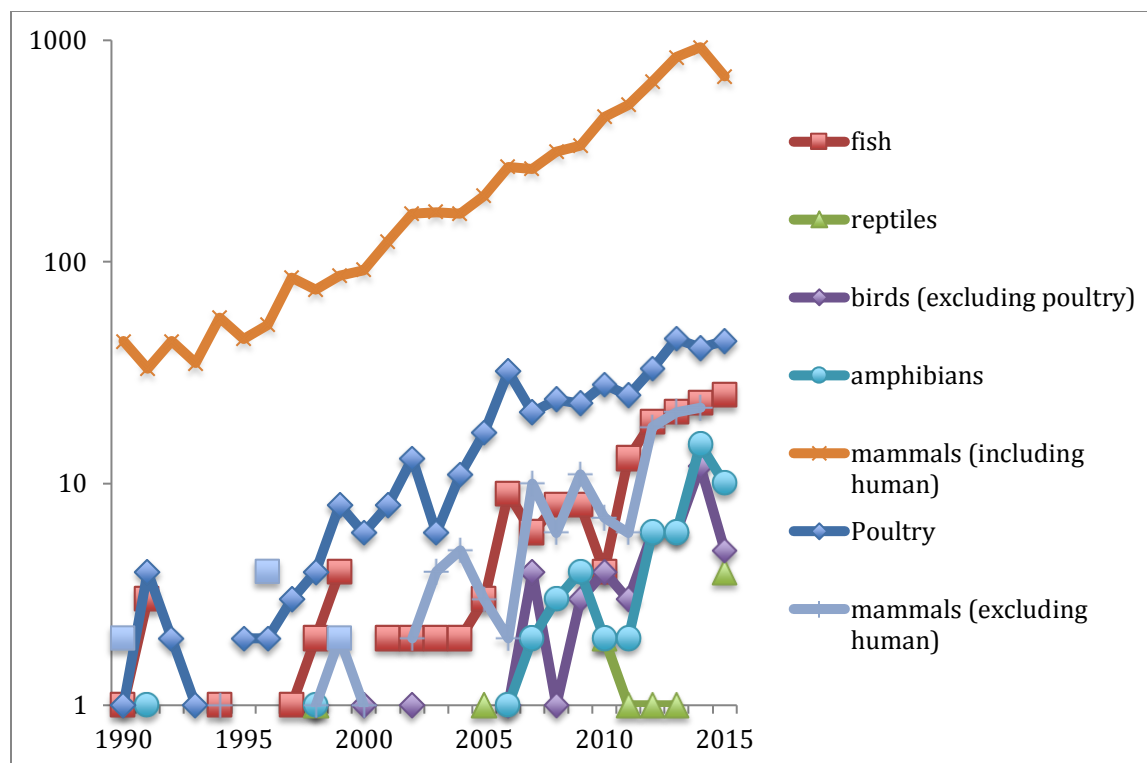
Figure S1. Increase in the number of microbiome studies for different classes of vertebrates over a 25 year period from 1990 to 2015.
Numbers were derived from a search of the Scopus database (www.scopus.com) for each of amphibians, birds, fish, reptiles, and mammals using the search terms "microbiome" and/or "bacteria" and "gut", and not "mouth" and not "blood". Mammals both including and excluding human studies are shown and domesticated poultry displayed as their own category. Because of an increased interest in that area, amphibians included the search term "skin". Publication number is shown on a log scale. Microbiome studies of mammals have increased at a fairly consistent exponential rate each year, whereas increases in studies of non-mammalian vertebrates have been more erratic and even combined fall substantially below those of mammals.
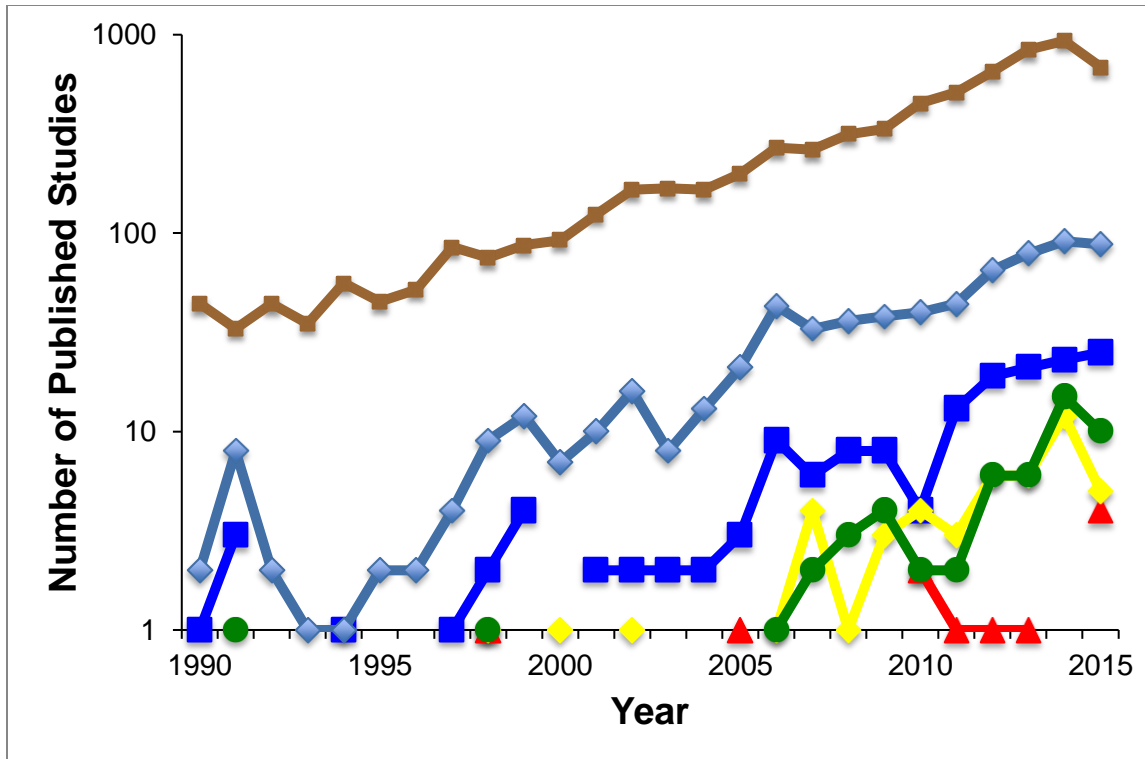
Figure S2. Increase in the number of microbiome studies for different classes of vertebrates over a 25 year period from 1990 to 2015.

Numbers were derived from a search of the Scopus database (www.scopus.com) for each of amphibians (green), birds (yellow), fish (blue), reptiles (red), all non mammalian vertebrates combined (light blue) and mammals (brown) using the search terms "microbiome" and/or "bacteria" and "gut", and not "mouth" and not "blood". Mammals included humans while for birds we excluded strictly domesticated poultry studies focused on pathogens. Because of an increased interest in that area, amphibians included the search term "skin". Publication number is shown on a log scale. Microbiome studies of mammals have increased at a fairly consistent exponential rate each year, whereas increases in studies of non-mammalian vertebrates have been more erratic and even combined fall substantially below those of mammals.
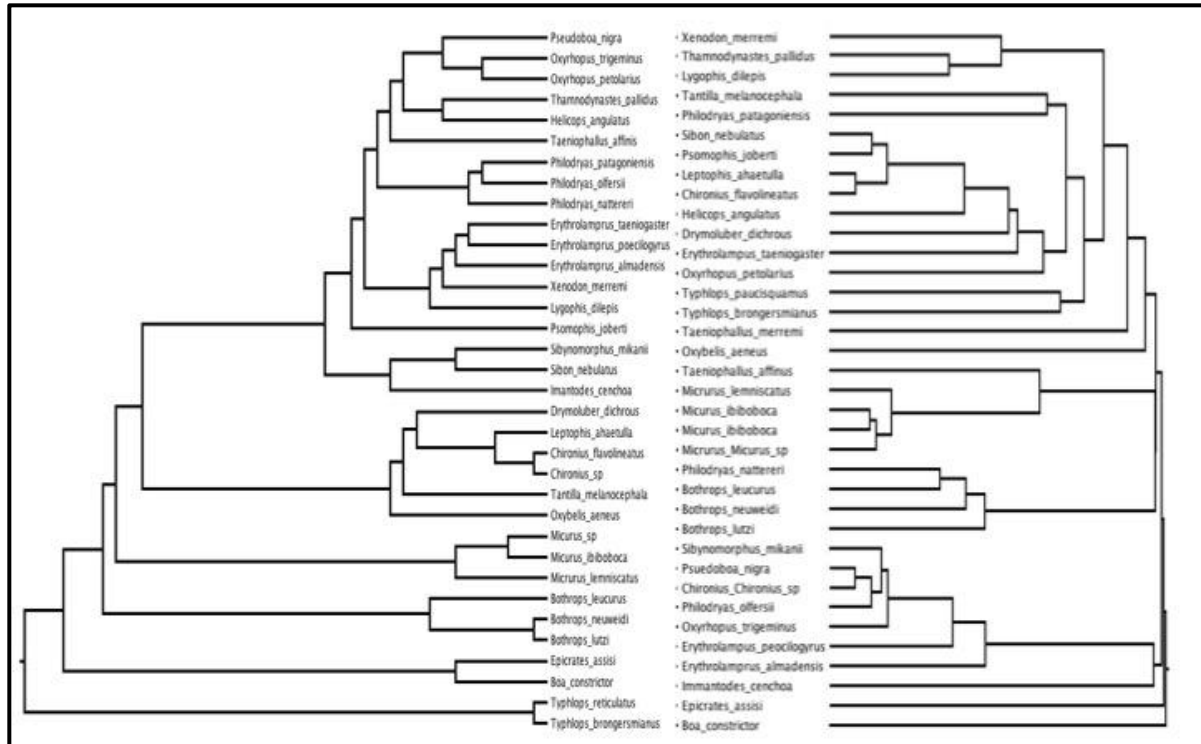
Supplementary Figure S1a. Comparison of host phylogeny (left) and UPGMA dendrogram of gut bacterial community structure (right) for our tropical snake community. Host phylogeny is based on Pyron *et al.* 2013 and dendrogram is based on UniFrac distance matrix.
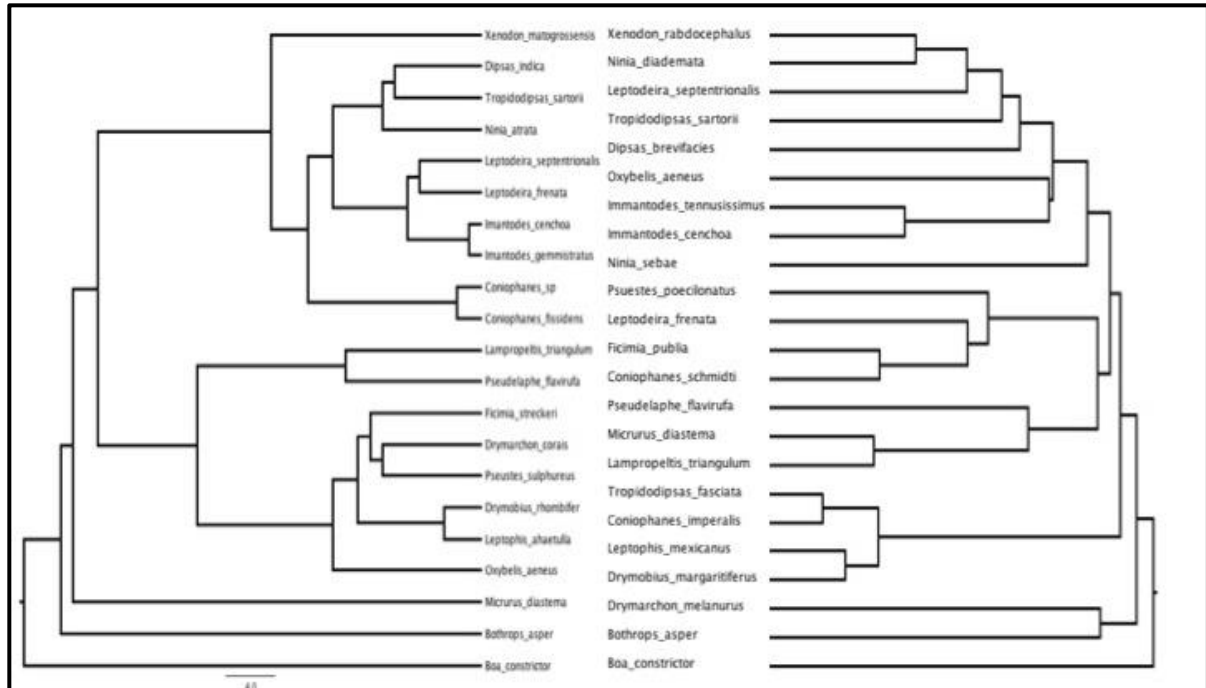
Supplementary Figure S1b. Comparison of host phylogeny (left) and UPGMA dendrogram of gut bacterial community structure (right) for our subtropical snake community. Host phyloeny is based on Pyron *et al.* 2013 and dendrogram is based on UniFrac distance matrix.

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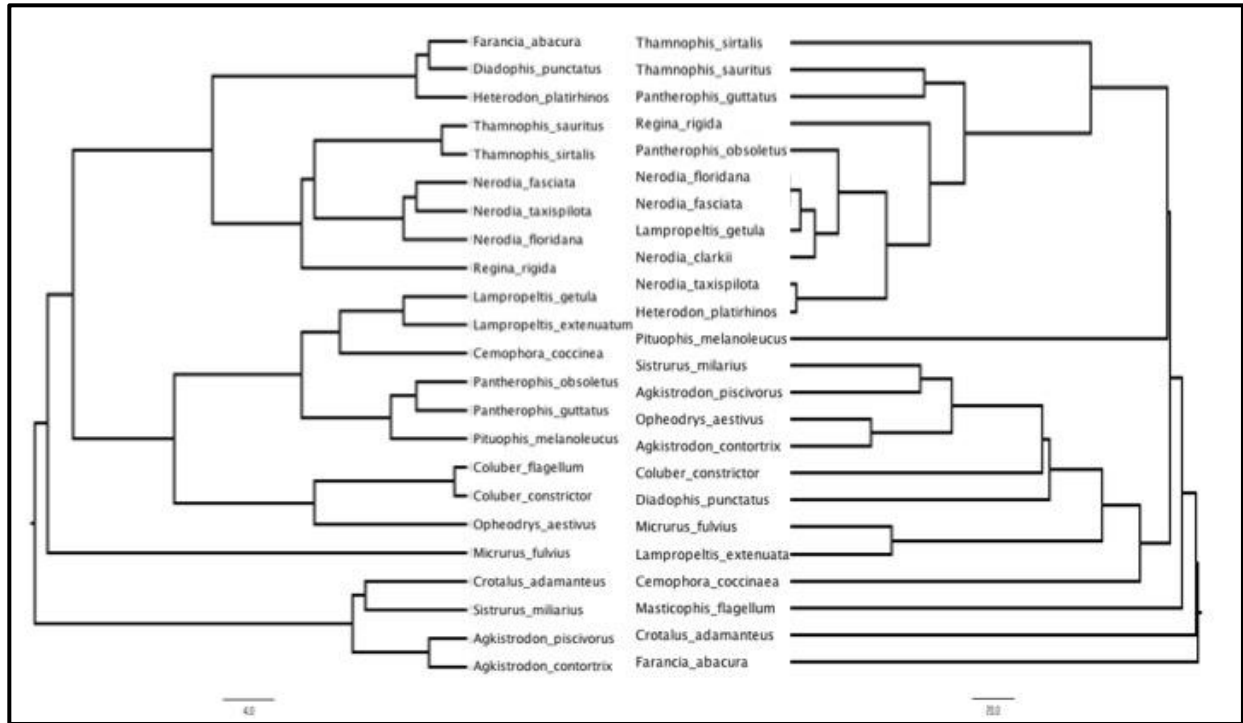
Supplementary Figure S1c. Comparison of host phylogeny (left) and UPGMA dendrogram of gut bacterial community structure (right) for our temperate snake community. Host phyloeny is based on Pyron *et al.* 2013 and dendrogram is based on UniFrac distance matrix.

Supplementary Figure S2. Comparison of host phylogeny and UPGMA dendrograms of gut bacterial community structure for our three snake communities. Host phyloeny is based on Pyron *et al.* 2013 and dendrogram is based on UniFrac distance matrix. A. Hypothesis of host family and subfamily relationships based on Pyron *et al.* 2013. B. Gut bacterial community structure of our tropical snake community. C. Gut bacterial community structure of our subtropical snake community. D. Gut bacterial community structure of our temperate snake community.

Table S1. Summary of host organism, location of microbiome, wild vs. captive, and number of publications from 229 published studies since 1990. C=captive, W=Wild

Organism	Technology used	Marker or 16S region	Type of sample	C or W	# of studies
Fish	454 pyrosequencing	16S	Feces	C	2
Fish	454 pyrosequencing	16S	Hindgut contents	C	1
Fish	454 pyrosequencing	V3	Hindgut contents	C	1
Fish	454 pyrosequencing	16S	Intestinal contents	C	1
Fish	454 pyrosequencing	V1-V2	Intestinal contents	C	1
Fish	454 pyrosequencing	V1-V3	Skin, olfactory tissue, anterior & posterior gut tissue	C	1
Fish	454 pyrosequencing	16S	Whole intestine	C	2
Fish	454 pyrosequencing	16S	Gut contents	W	1
Fish	pyrosequencing	V1-V3	Intestinal tissue	W&C	1
Fish	Cloning and SS	16S	Hindgut tissue	C	1
Fish	Cloning and SS	16S	Whole intestine	C	1
Fish	Cloning and SS	16S	Foregut tissue	W	1
Fish	Cloning and SS	16S	Intestinal mucosa	W	1
Fish	Cloning and SS & TRFLP	16S	Stomach, mid and hindgut contents	W	1
Fish	Cloning, PCR, DGGE	16S	Feces	W	1
Fish	Culture & DGGE		Foregut, midgut, hindgut tissue	C	1
Fish	Culture & DGGE		Whole intestine	C	1
Fish	Culture & DGGE		Hindgut contents	W	1
Fish	Culture & PCR	16S	Intestinal contents	C	1
Fish	Culture & PCR	16S	Whole gut	C	1
Fish	Culture & qPCR	16S	Feces	C	1
Fish	Culture & SS	16S	Feces	C	1
Fish	Culture & SS	16S	Foregut and Hindgut tissue	C	1
Fish	Culture & SS	V1, V3	Foregut and hindgut tissue	C	1
Fish	Culture & SS	16S	Foregut, midgut, hindgut tissue	C	2
Fish	Culture & SS	16S	Gill, gut and spleen tissue	C	1
Fish	Culture & SS	16S	GIT & contents	C	1

Fish	Culture & SS	16S	Hindgut tissue	C	1
Fish	Culture & SS	16S	Intestinal contents	C	1
Fish	Culture & SS	16S	Intestinal tissue	C	3
Fish	Culture & SS	16S	Midgut and hindgut tissue	C	1
Fish	Culture & SS	16S	Whole gut	C	1
Fish	Culture & SS	16S	Whole intestine	C	4
Fish	Culture & SS	16S	Hindgut contents	W	1
Fish	Culture & SS	16S	Intestinal contents	W	1
Fish	Culture & SS	16S	Whole intestine	W	3
Fish	Culture & SS	16S	Hindgut mucosa	W&C	1
Fish	Culture only		Feces	C	1
Fish	Culture only		Foregut and hindgut tissue	C	2
Fish	Culture only		Foregut, midgut, hindgut tissue	C	2
Fish	Culture only		Gill, osophegus, stomach and intestinal tissue	C	1
Fish	Culture only		Gut contents	C	1
Fish	Culture only		Gut mucosa	C	1
Fish	Culture only		Hindgut tissue	C	1
Fish	Culture only		Intestinal contents	C	1
Fish	Culture only		Intestinal mucosa	C	1
Fish	Culture only		Intestinal tissue	C	2
Fish	Culture only		Skin and stomach swab	C	1
Fish	Culture only		Whole intestine	C	8
Fish	Culture only		Foregut and hindgut contents	W	1
Fish	Culture only		Foregut, midgut, hindgut tissue	W	1
Fish	Culture only		Intestinal contents	W	2
Fish	Culture only		Intestinal mucosa	W	1
Fish	Culture only		Intestinal tissue	W	1
Fish	Culture only		Skin, stomach and intestine contents	W	1
Fish	Culture only		Stomach and intestine contents	W	1
Fish	Culture only		Skin, gill tissue, whole gut	W&C	1
Fish	Culture only		Whole larvae and hindgut tissue (adults)	C	1
Fish	Culture, cloning, PCR & SS	16S	Hindgut contents	W	1
Fish	Culture, PCR,	16S	Feces, gut tissue	C	1

	DGGE, SS				
Fish	Culture, PCR, DGGE, SS	V1, V3	Foregut, midgut, hindgut tissue	C	1
Fish	Culture, PCR, DGGE, SS	V3	Foregut, midgut, hindgut tissue	C	2
Fish	Culture, PCR, DGGE, SS	16S	Gut contents	C	1
Fish	Culture, PCR, DGGE, SS	16S	Intestinal contents	C	1
Fish	Culture, PCR, DGGE, SS	16S	Intestinal contents and mucosa	C	1
Fish	Culture, PCR, DGGE, SS	16S	Intestinal tissue	C	1
Fish	Culture, PCR, DGGE, SS	V3	Intestinal tissue	C	1
Fish	Culture, PCR, DGGE, SS	V3-V5	Pooled gut tissue	C	1
Fish	Culture, PCR, DGGE, SS	16S, ITS1, ITS4	Stomach and intestine contents, feces	C	1
Fish	Culture, PCR, DGGE, SS	16S	Whole intestine	C	1
Fish	Fluorescent imagery		Intestinal tissue and mucosa	C	1
Fish	Fluorescent imagery		Whole intestine	C	2
Fish	Fluorescent imagery,cloning & SS	16S	Whole intestine	C	1
Fish	Illumina HiSeq	16S & metagenomic	Foregut, midgut, hindgut, rectum tissue	C	1
Fish	Illumina HiSeq	16S	Whole intestine	C	1
Fish	Illumina HiSeq	RNA-seq	Whole intestine	C	1
Fish	Illumina HiSeq	16S & metagenomic	Whole intestine	C	1
Fish	Illumina HiSeq	V5	Whole intestine	C	1
Fish	Illumina HiSeq	V4	Skin and whole gut	W	1
Fish	Illumina MiSeq	V4	cecum tissue	C	1
Fish	Illumina MiSeq	V4	Foregut, midgut, hindgut tissue	C	1
Fish	Illumina MiSeq	V4	Gut tissue	C	1
Fish	Illumina MiSeq	16S	Whole intestine	C	1
Fish	Illumina MiSeq	V3-V4	Whole intestine	C	1
Fish	Illumina MiSeq	16S	Whole intestine	W	1
Fish	Illumina MiSeq	V4	Whole intestine	W&C	1
Fish	PCR & SS	16S	Feces	C	1
Fish	PCR & SS	16S	Hindgut tissue	C	1

Fish	PCR & SS	16S	Intestinal tissue	C	1
Fish	PCR & SS	V3	Intestinal tissue	C	1
Fish	PCR & SS	V3	Whole gut	C	1
Fish	PCR & SS	16S	Whole larvae	C	1
Fish	PCR &DGGE	16S	Hindgut contents	C	1
Fish	PCR &DGGE	16S	Intestinal tissue	C	1
Fish	PCR &DGGE	16S	Whole intestine	C	2
Fish	PCR &DGGE	V3	Whole intestine	C	1
Fish	PCR &DGGE	V6-V8	Hindgut contents	W&C	1
Fish	PCR, DGGE, 454 pyrosequencing	V6-V8	Hindgut contents	C	1
Fish	PCR, DGGE, 454 pyrosequencing	V1-V3	Intestinal contents and mucosa	C	1
Fish	PCR, DGGE, qPCR	16s	Foregut and hindgut tissue	C	1
Fish	PCR, DGGE, SS	V3	Intestinal tissue	C	1
Fish	PCR, DGGE, SS	V3	Hindgut and contents	C	1
Fish	PCR, DGGE, SS	V3	Feces	C	1
Fish	PCR, DGGE, SS	V3	Gut contents	C	1
Fish	PCR, DGGE, SS	V3	Intestinal contents	C	1
Fish	PCR, DGGE, SS	V3	Intestinal contents and mucosa	C	1
Fish	PCR, DGGE, SS	V3	Foregut and hindgut tissue	C	1
Fish	PCR, DGGE, SS	16S	Whole larvae and whole intestine (adults)	C	1
Fish	PCR, DGGE, SS, qPCR	V3	Whole larvae	C	1
Fish	PCR, TGGE, SS	16S	Hindgut contents	C	1
Fish	SEM		Foregut, midgut, hindgut tissue	C	1
Fish	SEM		epulo	W	1
Fish	SEM		Gut contents	W	1
Fish	SEM		Whole gut	W	1
Fish	SEM, Culture, PCR & SS	16S	Foregut, midgut, hindgut tissue	C	1
Frog	454 pyrosequencing	*L. hongkongensis*	Sectioned gut	C	1
Frog	454 pyrosequencing	V1-V2	Skin swab	W	1
Frog	454 pyrosequencing	V2	Skin swab	W	1
Frog	454 pyrosequencing	V2	Skin swab	W&C	1
Frog	Culture &	V4	Skin swab	W	1

	Illumina MiSeq				
Frog	Culture & SS	16S	Skin & clocal swab	C	1
Frog	Culture & SS	16S	Skin swab	C	1
Frog	Culture & SS	16S	Sectioned gut	W	1
Frog	Culture & SS	16S	Skin swab	W	6
Frog	Culture & SS	16S	Skin swab	W&C	1
Frog	Culture & SS, cDNA		Skin tissue	C	1
Frog	Culture only		Skin swab	C	6
Frog	Culture only		Skin swab	W	3
Frog	Culture only		Feces	W	1
Frog	Culture only		Clocal swab	W	1
Frog	Illumina HiSeq	V4	Skin swab	W	1
Frog	Illumina MiSeq	V4	Skin swab	W&C	1
Frog	PCR & DGGE	V3-V4	Skin swab	W&C	1
Frog	qPCR	*J. lividum*	Skin swab	C	1
Frog	TRLFP	16S	Skin swab	W&C	1
Frog	Illumina MiSeq	16S	Whole gut	C	1
Frog (tadpole)	Illumina MiSeq	16S	Whole gut	C	1
Salamander	454 pyrosequencing	V2	Skin swab	W	1
Salamander	454 pyrosequencing	V4	Skin swab	W	1
Salamander	454 pyrosequencing	V2	Skin swab	W&C	1
Salamander	Culture & DGGE	16S	Skin swab	W	1
Salamander	Culture & Illumina MiSeq	V4	Skin swab	W	1
Salamander	Culture & SS	*J. lividum*	Skin swab	C	1
Salamander	Culture & SS	*J. lividum*	Sectioned gut & contents	W	1
Salamander	Culture only		Skin swab	W	1
Salamander	Culture, PCR, DGGE, SS	16S	Skin swab	W	2
Salamander	Illumina HiSeq	V4	Skin swab	W	1
Salamander	Illumina HiSeq	V4	Skin swab	W	1
Salamander	Illumina MiSeq	V4	Skin swab	W&C	1
Salamander	PCR &DGGE	16S	Skin swab	W&C	1
Salamander	qPCR	16S	Skin swab	C	1
Lizard	454 pyrosequencing	16S	Feces	W	1
Lizard	Illumina MiSeq	V4	Colon tissue	C	1
Lizard	PCR &DGGE	16S	Feces	C	1
Snake	454	V2	Cecum, small and	C	1

	pyrosequencing		large intestine mucosa		
Snake	454 pyrosequencing	V4	Small intestine, large intestine and cloacal swabs	W	1
Snake	Cloning & SS, Ion Torrent	16S & metagenomic	Sectioned stomach, small and large intestine tissue	W	1
Snake	Culture only		Clocal swab	W	1
Snake	PCR &DGGE	16S	Feces	C	1
Snake	PCR, DGGE, SS	16S	Clocal swab	W	2
Tortoise	Illumina MiSeq	V4	Feces	W	1
Alligator	454 pyrosequencing	V1-V3	Tongue swab, esophagus tissue, stomach tissue, gastric juice, ileum tissue, duodenum tissue, colon tissue, fecal tissue	W&C	2
Bird	454 pyrosequencing	16S	Feces	C	1
Bird	454 pyrosequencing	V1-V3	Feces	W	2
Bird	454 pyrosequencing	V2	Feces	W	2
Bird	454 pyrosequencing	V3-V4	Feces	W	2
Bird	454 pyrosequencing	V3-V4	Cloacal swab	W	1
Bird	454 pyrosequencing	16S	Feces	W&C	1
Bird	454 pyrosequencing	16S	Crop & choana swabs, feces	W&C	1
Bird	454 pyrosequencing	V3-V4	Facial swabs and large intestine samples	W&C	1
Bird	Cloning and SS	16S	Feces	W	4
Bird	Cloning and SS	16S	crop	W	2
Bird	Cloning and SS	16S	Cloacal swab	W	2
Bird	Cloning and SS	16S	Cecum	W	2
Bird	Cloning, PCR & RFLP	16S	Feces, Cecum tissue	W	1
Bird	Culture only		Ejaculate swab	W	1
Bird	Culture only		Feces	W&C	1
Bird	Illumina MiSeq	V4	cecum tissue	C	1
Bird	Illumina MiSeq	V6	Intestinal tissue	W	1
Bird	Illumina MiSeq	V4-V5	Feces	W&C	1
Bird	PCR	16S	Feces	W&C	1

Supplementary Table S1. Metadata associated with samples used in this study. MS excel file "tableS1.xls".

Supplementary Table S2. Alpha diversity statistics for samples used in this study.

ID	S_{obs}	*Coverage*	Inverse Simpsons Index	S_{chao1}
TJCS1440_P_flavipunctata_Gekkonidae	4.860	0.999	1.016	5.098
TJCS1211_L_septentrionalis_Dipsadinae	10.745	0.998	1.859	12.063
TJCS1047_T_sartorii_Dipsadinae	7.333	0.998	2.334	9.449
TJCS1220_N_diademata_Dipsadinae	10.022	0.998	3.604	12.413
TJCS1193_X_rabdocephalus_Dipsadinae	15.474	0.997	5.268	17.721
TJCS763_L_frenata_Dipsadinae	8.156	0.997	2.375	11.120
TJCS998_P_flavirufa_Colubrinae	13.827	0.997	4.932	16.305
TJCS1393_T_striata_Scincidae	6.005	0.997	1.963	9.889
TJCS1030_I_tennuissimus_Dipsadinae	16.002	0.997	3.040	18.488
TJCS1189_X_rabdocephalus_Dipsadinae	17.470	0.996	3.452	21.358
TJCS1032_O_aeneus_Colubrinae	13.998	0.996	4.556	17.997
TJCS934_B_asper_Viperidae	12.477	0.996	2.302	17.229
KW2048_N_fasciata_Natricinae	9.806	0.996	2.125	14.543
TJCS762_P_poecilonatus_Colubrinae	8.745	0.996	2.108	13.925
TJCS184_P_obsoletus_Colubrinae	19.477	0.996	2.792	23.061
TJCS158RT_E_poecilogyrus_Dipsadinae	49.668	0.996	5.594	50.433
TJCS130RT_B_neuweidi_Viperidae	29.852	0.996	1.698	32.813
KW2005a_P_obsoletus_Colubrinae	14.970	0.995	2.640	20.198
TJCS1176_B_asper_Viperidae	24.121	0.995	8.597	28.972
TJCS161_C_adamanteus_Viperidae	18.230	0.995	4.646	24.899
TJCS31RTb_M_ibiboboca_Elapidae	56.949	0.995	31.684	59.633
TJCS128RT_O_aeneus_Colubrinae	9.347	0.995	1.195	17.738
TJCS786_L_triangulum_Colubrinae	14.690	0.995	2.769	22.214
TJCS159RT_M_aff_ibiboboca_Elapidae	8.941	0.995	1.048	16.318
TJCS134RT_unknown_RT537_Alethinophidia	28.171	0.995	9.320	36.829
TJCS155RT_A_brongersmianus_Typhlopidae	49.460	0.995	28.549	52.610
TJCS1425_C_rivae_Cordylidae	14.219	0.995	2.023	20.769
TJCS158_T_sirtalis_Natricinae	14.004	0.995	1.981	23.573
TJCS876_C_horridus_Viperidae	18.903	0.995	2.882	25.896
TJCS857_P_flavirufa_Colubrinae	10.484	0.995	1.392	17.939
KW1894_P_melanoleucus_Colubrinae	16.632	0.994	4.029	26.194
TJCS148RT_O_aeneus_Colubrinae	42.732	0.994	17.054	47.654
TJCS145_S_miliarius_Viperidae	23.677	0.994	7.623	30.325
TJCS162_N_fasciata_Natricinae	17.566	0.994	3.707	24.833

TJCS169_P_obsoletus_Colubrinae	15.719	0.994	3.976	23.854
TJCS185_P_obsoletus_Colubrinae	18.697	0.994	2.637	26.536
TJCS791_B_asper_Viperidae	24.296	0.994	5.241	32.637
TJCS933_C_imperalis_Dipsadinae	13.481	0.994	1.839	20.407
KW2156_L_getula_Colubrinae	30.164	0.994	5.929	35.216
TJCS152_S_miliarius_Viperidae	21.327	0.994	6.828	29.691
TJCS146_S_miliarius_Viperidae	20.798	0.994	8.078	27.952
TJCS631_D_margaritiferus_Colubrinae	31.919	0.994	7.293	36.228
TJCS1002_D_brevifacies_Dipsadinae	23.295	0.994	4.085	28.156
TJCS312_C_resimus_Viperidae	21.754	0.994	2.204	28.469
TJCS1221_I_cenchoa_Dipsadinae	22.158	0.994	4.452	29.402
TJCS30RTb_B_leucurus_Viperidae	60.530	0.993	20.247	65.126
TJCS1431_A_annectans_Agamidae	45.331	0.993	8.701	49.298
KW2006a_P_obsoletus_Colubrinae	26.947	0.993	4.696	34.606
TJCS1000_T_sartorii_Dipsadinae	45.736	0.993	3.013	50.747
TJCS1029_D_melanurus_Colubrinae	35.314	0.993	11.755	42.258
TJCS160_O_aestivus_Colubrinae	15.350	0.993	2.727	27.732
TJCS150_S_miliarius_Viperidae	18.538	0.993	3.394	29.509
TJCS159_M_flagellum_Colubrinae	16.652	0.993	1.761	27.673
TJCS90RT_A_brongersmianus_Typhlopidae	45.970	0.993	25.714	55.275
TJCS149RT_P_joberti_Dipsadinae	43.017	0.993	4.421	49.917
TJCS157RT_I_cenchoa_Dipsadinae	43.518	0.993	14.463	51.155
KW2028_P_obsoletus_Colubrinae	38.211	0.993	3.630	43.600
TJCS191_S_miliarius_Viperidae	24.106	0.992	4.283	32.443
TJCS647b_L_mexicanus_Colubrinae	28.475	0.992	3.279	34.870
TJCS82RTb_A_paucisquamus_Typhlopidae	39.026	0.992	1.635	44.620
TJCS820_C_imperalis_Dipsadinae	27.452	0.992	6.086	37.315
TJCS1157_F_abacura_Dipsadinae	19.797	0.992	1.841	32.740
TJCS1018_L_frenata_Dipsadinae	17.864	0.992	2.304	30.041
TJCS877_A_contortrix_Viperidae	29.335	0.992	6.683	38.057
TJCS54RT_A_paucisquamus_Typhlopidae	57.749	0.992	8.890	64.241
TJCS1191_A_biporcatus_Dactyloidae	27.403	0.992	4.245	38.222
KW2060_L_extenuata_Colubrinae	25.651	0.992	3.697	35.305
TJCS153RT_P_olfersii_Dipsadinae	52.715	0.992	21.350	59.364
TJCS544_T_balebicornutus_Chamaeleonidae	23.942	0.991	7.405	41.706
TJCS760_M_diastema_Elapidae	18.393	0.991	1.212	31.299
TJCS163_T_sauritus_Natricinae	19.623	0.991	3.241	34.920
KW2011_R_rigida_Natricinae	26.232	0.991	4.299	38.921
TJCS146RT_B_leucurus_Viperidae	64.125	0.991	10.819	71.380
TJCS439_L_fulginosus_Lamprophiidae	18.917	0.991	3.602	37.492
TJCS85RT_A_brongersmianus_Typhlopidae	64.507	0.991	15.165	72.218
TJCS25RTb_P_patagoniensis_Dipsadinae	83.040	0.991	29.767	89.207
TJCS150RT_H_angulatus_Dipsadinae	45.208	0.991	2.513	53.271

TJCS147_S_miliarius_Viperidae	25.061	0.991	2.946	35.591
TJCS24RTb_S_mikanii_Dipsadinae	71.667	0.991	26.115	78.851
TJCS141RT_P_olfersii_Dipsadinae	48.606	0.991	9.929	58.485
TJCS1007_D_brevifacies_Dipsadinae	23.839	0.991	1.241	35.871
TJCS1213_L_septentrionalis_Dipsadinae	28.173	0.991	6.097	42.478
TJCS1360_H_cf_flaviviridis_Gekkonidae	12.578	0.991	1.030	35.005
TJCS182_P_guttatus_Colubrinae	17.809	0.991	1.201	39.459
TJCS131RT_P_olfersii_Dipsadinae	33.900	0.990	1.783	48.538
KW1994b_N_fasciata_Natricinae	19.628	0.990	2.366	40.102
TJCS616_A_rodriguezii_Dactyloidae	24.289	0.990	2.856	39.340
TJCS166_C_constrictor_Colubrinae	28.143	0.990	4.792	43.373
TJCS1209_B_asper_Viperidae	37.076	0.990	4.395	50.896
TJCS1216_B_asper_Viperidae	23.026	0.990	4.674	41.917
TJCS53RT_B_leucurus_Viperidae	64.693	0.990	9.457	72.509
TJCS84RT_B_leucurus_Viperidae	72.688	0.990	12.763	79.782
TJCS1031_B_vittatus_Corytophanidae	25.518	0.990	4.477	42.979
TJCS168_D_puntatus_Dipsadinae	58.041	0.990	11.582	66.483
KW2045b_T_sauritus_Natricinae	34.468	0.990	3.463	49.207
TJCS345_B_arietans_Viperidae	45.216	0.990	1.605	54.373
TJCS62RT_A_paucisquamus_Typhlopidae	76.118	0.990	23.031	83.746
TJCS753_M_diastema_Elapidae	17.039	0.990	1.128	39.799
TJCS61RT_T_melanocephala_Colubrinae	70.813	0.990	9.001	79.263
KW2159b_N_taxisilota_Natricinae	26.883	0.990	2.350	35.788
TJCS189_N_fasciata_Natricinae	22.729	0.990	2.734	43.640
TJCS71RT_A_paucisquamus_Typhlopidae	73.183	0.989	5.477	80.823
TJCS190_T_sirtalis_Natricinae	26.125	0.989	3.343	45.878
TJCS285_P_battersbyi_Colubrinae	17.951	0.989	1.262	47.617
TJCS1192_X_rabdocephalus_Dipsadinae	49.763	0.989	10.456	58.109
TJCS165RT_M_aff_ibiboboca_Elapidae	54.794	0.989	3.298	67.121
TJCS1214_L_septentrionalis_Dipsadinae	35.411	0.989	9.851	52.823
TJCS144RT_E_poecilogyrus_Dipsadinae	38.318	0.989	1.475	47.353
TJCS384_P_aff_sibilans_Lamprophiidae	22.382	0.988	2.455	49.872
TJCS52RT_A_paucisquamus_Typhlopidae	80.012	0.988	16.038	89.826
TJCS186_T_sirtalis_Natricinae	30.609	0.988	2.731	47.686
TJCS34RTB_T_melanocephala_Colubrinae	52.982	0.988	15.500	68.227
TJCS23RT_B_leucurus_Viperidae	26.863	0.988	6.385	56.122
TJCS183_M_fulvis_Elapidae	22.016	0.988	2.922	43.648
TJCS440_B_arietans_Viperidae	29.652	0.988	3.345	51.021
TJCS137RT_M_aff_ibiboboca_Elapidae	25.865	0.988	4.281	45.636
KW1999b_N_fasciata_Natricinae	22.268	0.988	2.344	47.058
TJCS1378_X_wilmsi_Agamidae	21.580	0.988	1.802	46.349
TJCS387_N_olivacea_Natricinae	50.039	0.988	10.720	65.374
TJCS132RT_M_aff_ibiboboca_Elapidae	27.056	0.988	1.345	40.869

TJCS59RT_A_paucisquamus_Typhlopidae	68.167	0.988	11.765	77.863
TJCS139RT_Chironius_sp_Colubrinae	70.635	0.988	16.034	80.630
TJCS630_C_imperalis_Dipsadinae	30.236	0.988	6.277	56.518
TJCs11RT_T_melanocephala_Colubrinae	91.839	0.988	19.096	100.831
TJCS171_A_piscivorus_Viperidae	35.971	0.988	4.751	61.609
KW2157_L_getula_Colubrinae	52.810	0.987	12.680	66.583
TJCS133RT_O_trigeminus_Dipsadinae	24.818	0.987	2.106	42.182
TJCS161RT_M_aff_ibiboboca_Elapidae	22.932	0.987	2.504	48.598
TJCS51RT_A_paucisquamus_Typhlopidae	83.546	0.987	12.557	93.288
TJCS170_C_constrictor_Colubrinae	38.339	0.987	10.056	62.286
KW2037_N_fasciata_Natricinae	43.106	0.987	3.608	56.889
TJCS1255_C_canninus_Boidae	39.788	0.987	5.550	55.537
KW2038_N_clarkii_Natricinae	33.901	0.987	7.467	53.093
TJCS464_Naja_haje_Elapidae	46.927	0.987	9.368	64.698
TJCS174_O_attenuatus_Anguidae	28.564	0.987	2.978	54.682
TJCS113RT_A_brongersmianus_Typhlopidae	61.182	0.987	6.704	73.771
TJCS349_C_laevigatus_Chamaeleonidae	107.219	0.987	22.794	114.781
TJCS50RT_M_ibiboboca_Elapidae	71.369	0.987	6.076	81.591
TJCS666b_T_fasciata_Dipsadinae	35.604	0.987	5.031	50.573
TJCS2RT_E_almadensis_Dipsadinae	22.564	0.987	3.575	57.397
TJCS167_C_constrictor_Colubrinae	71.868	0.986	14.615	82.822
TJCS72RT_E_poecilogyrus_Dipsadinae	79.961	0.986	4.596	88.823
TJCS143RT_B_leucurus_Viperidae	86.200	0.986	9.731	95.892
TJCS105RTb_P_olfersii_Dipsadinae	79.490	0.986	20.961	94.030
TJCS178_A_piscivorus_Viperidae	42.191	0.986	7.232	63.859
TJCS433_P_aff_sibilans_Lamprophiidae	27.210	0.986	2.210	52.996
TJCS123RT_H_angulatus_Dipsadinae	90.661	0.986	16.434	96.604
TJCS122RT_E_taeniogaster_Dipsadinae	31.990	0.986	3.482	49.035
TJCS124RT_A_brongersmianus_Typhlopidae	46.346	0.986	5.280	61.089
TJCS461_P_punctulatus_Lamprophiidae	40.544	0.986	3.103	61.422
TJCS79RT_C_flavolineatus_Colubrinae	24.179	0.986	1.415	49.506
TJCS463_B_arietans_Viperidae	100.079	0.986	13.795	108.567
TJCS1208_N_diademata_Dipsadinae	49.269	0.986	9.423	69.240
KW2040_N_clarkii_Natricinae	45.051	0.986	7.451	59.117
TJCS822_L_triangulum_Colubrinae	27.462	0.986	3.009	52.415
TJCS99RT_M_lemniscatus_Elapidae	40.627	0.986	2.974	57.682
TJCS4RT_B_lutzi_Viperidae	32.304	0.986	4.660	55.704
TJCS47RT_P_nattereri_Dipsadinae	28.572	0.986	2.643	53.371
TJCS629b_C_imperalis_Dipsadinae	35.703	0.986	2.914	49.705
TJCS517b_T_affinis_Chamaeleonidae	26.032	0.985	2.334	54.087
TJCS92RTb_S_mikanii_Dipsadinae	26.058	0.985	2.474	52.861
TJCS1386_A_guenterpetersi_Agamidae	57.195	0.985	7.702	72.541
TJCS1182_C_cristatus_Corytophanidae	33.127	0.985	5.484	61.583

TJCS89RT_M_ibiboboca_Elapidae	60.631	0.985	3.406	75.363
KW2051_C_coccinaea_Colubrinae	49.151	0.985	6.735	64.060
TJCS96RT_M_ibiboboca_Elapidae	28.579	0.985	2.266	54.348
TJCS57RT_O_aeneus_Colubrinae	70.234	0.985	7.258	81.054
TJCS458_P_aff_sibilans_Lamprophiidae	26.329	0.985	2.774	58.964
TJCS347_C_laevigatus_Chamaeleonidae	104.326	0.985	17.738	113.023
TJCS1456_H_macropholis_Gekkonidae	45.825	0.985	8.686	82.222
TJCS181_A_contortrix_Viperidae	42.744	0.984	3.641	69.960
TJCS46RT_P_patagoniensis_Dipsadinae	78.131	0.984	6.363	90.721
TJCS114RT_A_paucisquamus_Typhlopidae	62.210	0.984	3.169	77.378
KW1996_N_fasciata_Natricinae	52.092	0.984	4.582	69.613
TJCS67RT_B_leucurus_Viperidae	93.729	0.984	23.908	104.453
TJCS143_N_sipedon_Natracinae	53.107	0.984	10.919	70.826
TJCS376_B_arietans_Viperidae	49.019	0.984	4.081	58.535
TJCS137_P_guttatus_Colubrinae	32.679	0.984	4.286	62.560
KW2029_P_obsoletus_Colubrinae	95.915	0.984	23.063	105.507
TJCS467_P_aff_sibilans_Lamprophiidae	31.252	0.983	2.319	66.272
TJCS1188_X_rabdocephalus_Dipsadinae	38.929	0.983	5.934	75.584
TJCS1495_V_albigularis_Varanidae	57.819	0.983	6.832	75.678
TJCS293_C_hotamboeia_Colubrinae	79.248	0.983	5.866	93.566
KW2050b_R_rigida_Natricinae	26.430	0.983	2.277	65.717
TJCS142_N_sipedon_Natracinae	31.715	0.983	2.354	65.422
TJCS91RT_P_patagoniensis_Dipsadinae	31.036	0.983	1.823	54.157
TJCS292_C_hotamboeia_Colubrinae	35.584	0.983	3.111	58.212
TJCS74RT_E_assisi_Boidae	39.590	0.983	3.145	71.520
TJCS1445_Panaspis_sp_Scincidae	31.359	0.983	3.289	62.893
TJCS377_C_laevigatus_Chamaeleonidae	30.050	0.982	1.517	78.191
TJCS100RT_B_leucurus_Viperidae	36.360	0.982	3.795	70.649
TJCS87RT_I_cenchoa_Dipsadinae	38.486	0.982	1.241	56.491
TJCS95RT_C_flavolineatus_Colubrinae	41.854	0.982	3.416	73.959
TJCS290_N_melanoleuca_Elapidae	45.264	0.982	2.655	78.189
TJCS64RT_B_leucurus_Viperidae	42.271	0.982	2.386	66.655
TJCS1450_L_keniensis_Gekkonidae	55.985	0.982	13.229	86.797
AMS525_U_superciliosus_Tropiduridae	30.428	0.981	2.337	69.317
TJCS135RT_M_lemniscatus_Elapidae	44.678	0.981	1.329	62.336
TJCS444_P_aff_sibilans_Lamprophiidae	62.810	0.981	6.449	79.502
TJCS36RTb_S_nebulatus_Dipsadinae	107.958	0.981	51.326	123.858
TJCS613_C_schmidti_Dipsadinae	50.586	0.981	5.836	75.269
TJCS112RT_C_flavolineatus_Colubrinae	56.518	0.981	5.070	79.111
TJCS93RTb_B_leucurus_Viperidae	66.019	0.981	3.032	82.227
TJCS129RTb_A_brongersmianus_Typhlopidae	98.540	0.981	16.583	113.314
TJCS145RT_B_leucurus_Viperidae	78.861	0.981	6.468	93.177
TJCS553_A_aff_cyanogaster_Agamidae	116.810	0.981	23.222	130.110

TJCS120RT_X_merremi_Dipsadinae	54.908	0.981	3.149	74.547
AMS534_B_atrox_Viperidae	44.855	0.980	8.027	87.081
TJCS173_A_piscivorus_Viperidae	43.903	0.980	1.396	68.773
TJCS434_L_fulginosus_Lamprophiidae	42.385	0.980	4.423	80.633
TJCS376b_B_arietans_Viperidae	44.205	0.980	3.929	71.213
TJCS108RTb_B_leucurus_Viperidae	94.205	0.980	5.164	109.213
TJCS63RT_O_trigeminus_Dipsadinae	48.158	0.980	1.895	70.073
TJCS180_A_contortrix_Viperidae	65.986	0.980	5.021	81.755
TJCS383_P_aff_sibilans_Lamprophiidae	37.448	0.979	1.699	81.402
TJCS140RT_H_angulatus_Dipsadinae	73.280	0.979	4.107	93.957
TJCS583_C_dilepis_Chamaeleonidae	119.685	0.979	25.310	135.264
TJCS462_A_microlepidota_Atractaspidae	93.203	0.979	11.338	106.525
TJCS138RT_Micrurus_sp_Elapidae	63.289	0.979	8.801	88.746
TJCS119RT_P_nigra_Dipsadinae	77.539	0.979	11.866	99.229
TJCS764_F_publia_Colubrina	65.314	0.978	4.697	85.553
TJCS1027KWb_N_fasciata_Natricinae	36.210	0.978	2.396	81.843
TJCS48RT_H_angulatus_Dipsadinae	50.764	0.978	1.339	71.783
TJCS929_D_melanurus_Colubrinae	50.830	0.978	4.025	76.956
TJCS76RT_H_angulatus_Dipsadinae	53.796	0.978	7.484	92.310
TJCS101RT_S_mikanii_Dipsadinae	46.241	0.978	1.836	83.525
TJCS154RTb_M_lemniscatus_Elapidae	61.070	0.978	1.814	77.802
TJCS142RT_B_leucurus_Viperidae	35.486	0.978	1.626	84.137
TJCS466_L_fulginosus_Lamprophiidae	36.194	0.977	1.976	82.674
TJCS78RT_P_nigra_Dipsadinae	84.333	0.977	6.996	102.774
TJCS160RT_O_trigeminus_Dipsadinae	100.291	0.977	5.186	115.821
TJCS332_B_arietans_Viperidae	86.890	0.977	3.004	100.938
TJCS26RTb_Chironius_sp_Colubrinae	44.949	0.977	1.814	73.636
TJCS81RT_E_assisi_Boidae	38.149	0.977	1.801	76.346
TJCS785_C_schmidti_Dipsadinae	59.669	0.977	5.888	88.039
TJCS43RT_H_angulatus_Dipsadinae	42.487	0.977	1.361	76.106
TJCS141_A_piscivorus_Viperidae	53.263	0.977	4.342	92.996
TJCS1411_H_cf_macropholis_Gekkonidae	66.297	0.977	6.017	98.733
TJCS386_N_olivacea_Natricinae	32.617	0.977	2.243	101.251
TJCS68RT_L_dilepis_Dipsadinae	65.534	0.977	2.916	84.989
TJCS795_B_vittatus_Corytophanidae	48.136	0.977	3.301	80.302
TJCS928_B_constrictor_Boidae	48.640	0.977	2.901	87.612
TJCS27RT_A_brongersmianus_Typhlopidae	64.824	0.977	5.622	94.502
TJCS116RT_E_taeniogaster_Dipsadinae	68.401	0.976	3.941	103.241
TJCS60RT_A_paucisquamus_Typhlopidae	62.135	0.976	6.936	85.193
TJCS162RT_Micrurus_sp_Elapidae	47.046	0.976	1.568	78.713
TJCS3RTb_E_assisi_Boidae	77.918	0.976	11.173	102.398
TJCS13RT_H_angulatus_Dipsadinae	40.273	0.976	1.272	83.174
TJCS289_P_battersbyi_Colubrinae	54.943	0.976	2.116	82.702

TJCS88RT_P_nigra_Dipsadinae	79.750	0.976	23.822	120.345
TJCS118RT_H_angulatus_Dipsadinae	79.551	0.976	8.707	100.107
TJCS70RT_A_paucisquamus_Typhlopidae	54.214	0.976	2.146	80.316
TJCS548_T_harennae_Chamaeleonidae	51.401	0.976	3.902	78.424
TJCS435_B_arietans_Viperidae	45.961	0.976	4.359	96.252
TJCS384b_P_aff_sibilans_Lamprophiidae	61.565	0.976	3.391	102.805
TJCS425_B_arietans_Viperidae	46.025	0.975	4.276	89.132
TJCS58RT_H_angulatus_Dipsadinae	95.171	0.975	8.791	119.975
TJCS540b_T_balebicornutus_Chamaeleonidae	47.938	0.975	3.892	93.488
TJCS6RT_M_lemniscatus_Elapidae	44.557	0.975	1.405	83.754
TJCS12RT_H_angulatus_Dipsadinae	53.136	0.975	2.408	98.039
TJCS65RT_B_leucurus_Viperidae	52.605	0.975	2.010	85.416
TJCS784_L_frenata_Dipsadinae	67.159	0.975	11.810	105.521
TJCS49RT_H_angulatus_Dipsadinae	71.979	0.975	2.329	95.714
KW2003b_P_guttatus_Colubrinae	47.136	0.975	2.653	89.199
TJCS24RT_S_mikanii_Dipsadinae	101.116	0.975	14.000	117.819
TJCS499_A_aff_atricolis_Agamidae	47.272	0.975	2.199	87.669
TJCS77RT_M_aff_ibiboca_Elapidae	56.029	0.975	2.393	88.953
TJCS14RT_A_paucisquamus_Typhlopidae	79.847	0.975	6.351	100.057
TJCS999_N_sebae_Dipsadinae	66.562	0.974	7.109	102.178
TJCS250_P_battersbyi_Colubrinae	58.473	0.974	3.010	84.867
TJCS56RT_H_angulatus_Dipsadinae	95.653	0.974	6.003	117.968
TJCS568_A_lunulatus_Lamprophiidae	43.775	0.974	2.666	96.218
TJCS509_A_aff_minutus_Agamidae	41.260	0.974	1.330	93.701
TJCS125RTb_B_leucurus_Viperidae	85.280	0.974	4.026	105.982
TJCS630b_C_imperalis_Dipsadinae	54.407	0.974	3.740	103.606
TJCS8RT_T_affinus_Dipsadinae	79.716	0.974	4.495	100.884
TJCS152RTb_O_trigeminus_Dipsadinae	48.032	0.974	2.304	91.139
TJCS98RT_H_angulatus_Dipsadinae	48.165	0.973	1.638	100.708
TJCS21RT_M_aff_ibiboca_Elapidae	45.458	0.973	1.664	97.467
TJCS83RT_O_trigeminus_Dipsadinae	49.205	0.973	2.300	93.696
TJCS10RT_M_aff_ibiboca_Elapidae	47.801	0.973	2.230	98.847
TJCS37RT_B_leucurus_Viperidae	49.425	0.973	1.632	94.849
TJCS382_P_aff_sibilans_Lamprophiidae	69.821	0.973	11.440	104.941
TJCS30RT_B_leucurus_Viperidae	110.707	0.973	11.769	129.889
TJCS73RT_B_leucurus_Viperidae	118.255	0.973	25.850	138.413
TJCS135RTb_M_lemniscatus_Elapidae	57.874	0.972	5.022	101.417
TJCS579_C_gracilis_Chamaeleonidae	118.855	0.972	15.400	141.112
KW2008b_N_fasciata_Natricinae	58.472	0.972	2.662	96.592
TJCS643b_L_frenata_Dipsadinae	49.593	0.972	5.075	104.345
TJCS127RT_Chironius_RT528_Colubrinae	52.102	0.972	2.562	98.039
TJCS34RT_T_melanocephala_Colubrinae	83.667	0.972	8.634	105.039
TJCS617b_D_margaritiferus_Colubrinae	55.796	0.972	3.888	101.244

TJCS106RTb_S_mikanii_Dipsadinae	54.241	0.972	2.441	100.495
TJCS588_V_albigularis_Varanidae	72.990	0.972	11.386	113.872
TJCS25RT_P_patagoniensis_Dipsadinae	111.561	0.971	13.484	131.507
KW2039b_N_clarkii_Natricinae	58.267	0.971	2.606	98.853
KW2026a_P_obsoletus_Colubrinae	55.834	0.971	2.460	100.832
TJCS1166_C_scurrulus_Colubrinae	58.912	0.971	4.204	102.636
TJCS1026KWb_L_getula_Colubrinae	52.011	0.971	2.323	100.930
KW2041b_N_clarkii_Natricinae	62.127	0.971	3.208	109.264
AMS526_S_compressus_Dipsadinae	59.452	0.971	2.572	102.294
KW2002b_T_sauritus_Natricinae	51.950	0.971	2.185	101.221
TJCS538_T_harennae_Chamaeleonidae	77.486	0.971	2.630	103.684
TJCS117RT_P_nattereri_Dipsadinae	95.403	0.971	23.918	123.085
TJCS109RT_B_leucurus_Viperidae	68.817	0.970	6.302	102.765
TJCS1170_C_scurrulus_Colubrinae	59.125	0.970	3.178	104.666
TJCS348_C_laevigatus_Chamaeleonidae	72.322	0.970	4.824	99.383
KW1997b_T_sauritus_Natricinae	54.864	0.970	2.535	101.163
AMS536_U_superciliosus_Tropiduridae	54.034	0.970	2.868	107.665
TJCS110RT_B_leucurus_Viperidae	74.390	0.970	7.158	111.862
TJCS1010_C_schmidti_Dipsadinae	57.879	0.970	2.072	98.280
TJCS147RTb_E_poecilogyrus_Dipsadinae	55.609	0.970	2.508	110.816
TJCS69RT_H_angulatus_Dipsadinae	66.925	0.970	1.939	103.204
TJCS28RT_E_poecilogyrus_Dipsadinae	79.118	0.970	10.166	125.584
TJCS9RT_L_ahaetulla_Colubrinae	106.201	0.970	13.836	128.785
KW2158b_H_platyrhinos_Dipsadinae	52.894	0.970	2.683	102.953
TJCS1498_Latastia_cf_doriai_Lacertidae	50.526	0.970	3.711	132.576
AMS548_O_fulgidis_Colubrinae	57.004	0.970	2.423	103.180
AMS528_E_cenchria_Boidae	60.011	0.969	3.136	110.598
TJCS42RT_H_angulatus_Dipsadinae	61.365	0.969	9.243	120.801
KW1998b_T_sirtalis_Natricinae	53.860	0.969	2.366	103.474
TJCS389_P_aff_sibilans_Lamprophiidae	62.259	0.969	1.538	94.698
TJCS115RT_D_dichrous_Colubrinae	92.715	0.969	10.893	122.819
TJCS102RTb_P_nigra_Dipsadinae	59.079	0.969	2.695	108.402
AMS535_U_superciliosus_Tropiduridae	56.848	0.969	3.106	114.710
TJCS937_D_brevifacies_Dipsadinae	75.289	0.968	7.534	112.693
TJCS107RTb_P_olfersii_Dipsadinae	58.626	0.968	2.400	109.644
TJCS130RTb_B_neuweidi_Viperidae	89.892	0.968	2.572	114.860
TJCS15RT_T_melanocephala_Colubrinae	90.188	0.968	18.825	129.406
TJCS518_T_affinis_Chamaeleonidae	100.634	0.968	3.890	125.732
KW2009a_N_fasciata_Natricinae	67.285	0.968	3.664	112.214
AMS520_U_superciliosus_Tropiduridae	64.366	0.968	3.324	113.085
TJCS26RT_Chironius_sp_Colubrinae	55.780	0.968	1.968	108.867
TJCS1264_C_hortulanus_Boidae	61.499	0.968	3.353	115.007
TJCS123RTb_H_angulatus_Dipsadinae	62.909	0.967	2.695	111.634

TJCS5RT_M_ibiboboca_Elapidae	61.513	0.967	1.960	112.847
TJCS1300_B_atrox_Viperidae	70.698	0.967	4.772	118.979
TJCS97RT_M_lemniscatus_Elapidae	48.682	0.967	2.879	132.802
TJCS381_V_niloticus_Varanidae	128.243	0.967	14.214	154.662
TJCS29RT_S_nebulatus_Dipsadinae	103.893	0.967	4.857	130.636
TJCS104RTb_O_trigeminus_Dipsadinae	61.791	0.966	2.636	115.420
AMS555_L_annulata_Dipsadinae	70.197	0.966	2.680	117.507
KW2027b_P_obsoletus_Colubrinae	66.037	0.966	3.009	115.988
TJCS1437_H_robustus_Gekkonidae	67.703	0.966	1.655	113.632
TJCS1286_C_scurrulus_Colubrinae	72.267	0.966	3.339	119.798
TJCS158RTb_E_poecilogyrus_Dipsadinae	69.494	0.966	3.979	124.628
TJCS1028KWb_N_floridana_Natricinae	67.067	0.965	3.679	120.353
TJCS35RT_L_ahaetulla_Colubrinae	66.675	0.965	1.744	107.481
KW2001a_N_fasciata_Natricinae	70.314	0.965	3.785	121.795
TJCS1499_Latastia_cf_doriai_Lacertidae	77.148	0.965	7.735	130.677
TJCS40RT_E_taeniogaster_Dipsadinae	67.535	0.965	5.642	134.593
TJCS1251_U_superciliosus_Tropiduridae	78.191	0.965	4.662	128.859
TJCS80RT_P_patagoniensis_Dipsadinae	114.848	0.964	24.020	151.497
TJCS7RT_T_pallidus_Dipsadinae	105.706	0.964	5.123	133.571
AMS501_P_neuwiedii_Dipsadinae	78.938	0.964	4.837	129.601
AMS549_C_hortulanus_Boidae	66.449	0.964	2.738	126.223
KW1995b_N_fasciata_Natricinae	69.848	0.964	3.508	126.471
TJCS111RT_A_brongersmianus_Typhlopidae	74.800	0.964	7.466	136.976
TJCS1253_C_scurrulus_Colubrinae	78.568	0.963	3.944	129.446
TJCS660b_D_margaritiferus_Colubrinae	75.511	0.963	4.023	125.475
TJCS66RT_P_olfersii_Dipsadinae	122.191	0.963	14.663	155.399
TJCS18RT_A_paucisquamus_Typhlopidae	87.291	0.962	18.252	145.294
TJCS1165_C_caninus_Boidae	77.481	0.962	3.634	134.719
TJCS575_C_dilepis_Chamaeleonidae	92.691	0.962	2.405	129.570
TJCS45RT_H_angulatus_Dipsadinae	89.831	0.961	3.080	132.619
TJCS44RT_H_angulatus_Dipsadinae	116.513	0.961	11.222	154.449
TJCS55RT_H_angulatus_Dipsadinae	100.670	0.960	6.165	142.695
TJCS39RT_X_merremi_Dipsadinae	93.347	0.959	8.450	154.109
TJCS1288_A_fuliginosa_Amphisbaenidae	80.521	0.959	3.512	145.753
TJCS121RT_H_angulatus_Dipsadinae	99.360	0.959	5.390	147.802
TJCS126RTb_A_brongersmianus_Typhlopidae	126.450	0.959	13.481	162.694
TJCS586_A_aff_cyanogaster_Agamidae	144.698	0.959	13.380	180.476
TJCS554_A_aff_cyanogaster_Agamidae	101.237	0.958	2.279	139.441
TJCS827_N_sebae_Dipsadinae	84.435	0.958	8.469	173.379
TJCS390_P_aff_sibilans_Lamprophiidae	76.643	0.957	8.047	146.372
TJCS388_P_aff_sibilans_Lamprophiidae	90.864	0.957	4.616	140.350
TJCS16RT_A_paucisquamus_Typhlopidae	109.830	0.957	4.810	148.664
TJCS1470_A_cf_atricollis_Agamidae	111.454	0.957	19.798	148.275

TJCS792_B_vittatus_Corytophanidae	125.413	0.956	25.870	171.896
TJCS1292_N_rudis_Gymnopthalmidae	86.978	0.956	7.342	179.719
TJCS19RT_P_nattereri_Dipsadinae	93.528	0.955	3.637	155.273
KW2160b_N_fasciata_Natricinae	86.215	0.955	3.958	161.969
TJCS825_Unknown	98.423	0.954	2.394	158.880
TJCS17RT_O_trigeminus_Dipsadinae	85.207	0.954	3.909	165.717
TJCS426_B_arietans_Viperidae	92.557	0.953	6.371	155.410
TJCS510_A_aff_minutus_Agamidae	120.465	0.951	22.629	188.697
TJCS41RT_E_taeniogaster_Dipsadinae	119.561	0.948	12.245	177.858
TJCS3RT_E_assisi_Boidae	116.474	0.947	13.866	180.970
TJCS505_A_zonurus_Agamidae	163.015	0.947	20.557	208.957
TJCS38RT_O_petolarius_Dipsadinae	91.234	0.947	9.567	192.003
TJCS22RT_B_constrictor_Boidae	102.952	0.940	2.518	210.281
TJCS36RT_S_nebulatus_Dipsadinae	191.626	0.934	34.800	252.857
TJCS1RT_L_dilepis_Dipsadinae	116.490	0.933	3.076	212.123
TJCS497_A_aff_atricolis_Agamidae	161.364	0.931	29.482	251.974
TJCS20RT_H_angulatus_Dipsadinae	153.471	0.931	11.292	233.638
TJCS584_A_aff_cyanogaster_Agamidae	173.750	0.918	21.065	289.104

Supplementary Table S3. Metadata associated with samples used in this study, excel file "tableS3.xls".

Supplementary Table S4. Alpha diversity statistics for samples used in this study.

ID	S_{obs}	*Coverage*	Inverse Simpsons Index	S_{chao1}
TJCS1211_L_septentrionalis_Dipsadinae	10.82	0.998	1.98	12.07
TJCS1047_T_sartorii_Dipsadinae	7.53	0.998	2.43	9.84
TJCS1220_N_diademata_Dipsadinae	10.24	0.998	3.78	12.84
TJCS1193_X_rabdocephalus_Dipsadinae	15.70	0.997	5.61	18.30
TJCS998_P_flavirufa_Colubrinae	13.99	0.997	5.16	16.52
TJCS763_L_frenata_Dipsadinae	8.46	0.997	2.46	12.08
TJCS1030_I_tennuissimus_Dipsadinae	16.20	0.997	3.25	18.62
TJCS1189_X_rabdocephalus_Dipsadinae	17.68	0.997	3.78	21.68
TJCS934_B_asper_Viperidae	12.59	0.996	2.48	17.35
TJCS1032_O_aeneus_Colubrinae	14.24	0.996	4.85	18.38
TJCS762_P_poecilonatus_Colubrinae	8.87	0.996	2.16	14.14
TJCS130RT_B_neuweidi_Viperidae	30.21	0.996	1.81	33.13
KW2005a_P_obsoletus_Colubrinae	15.22	0.996	2.85	20.61
TJCS31RTb_M_ibiboboca_Elapidae	57.18	0.996	33.96	59.51
TJCS1176_B_asper_Viperidae	24.39	0.995	9.13	29.54
TJCS161_C_adamanteus_Viperidae	18.50	0.995	5.07	25.35
TJCS128RT_O_aeneus_Colubrinae	9.46	0.995	1.24	17.58
TJCS159RT_M_aff_ibiboboca_Elapidae	9.17	0.995	1.07	16.69
TJCS786_L_triangulum_Colubrinae	15.18	0.995	2.98	23.09
TJCS857_P_flavirufa_Colubrinae	10.77	0.995	1.46	18.61
TJCS158_T_sirtalis_Natricinae	14.48	0.995	2.12	25.12
TJCS148RT_O_aeneus_Colubrinae	43.14	0.995	18.98	47.74
KW1894_P_melanoleucus_Colubrinae	16.85	0.995	4.30	26.69
TJCS185_P_obsoletus_Colubrinae	18.88	0.994	2.77	27.08
TJCS145_S_miliarius_Viperidae	24.04	0.994	8.15	30.99
TJCS933_C_imperalis_Dipsadinae	13.74	0.994	1.93	20.80
TJCS1002_D_brevifacies_Dipsadinae	23.61	0.994	4.44	27.96
TJCS791_B_asper_Viperidae	24.62	0.994	5.66	32.65
KW2156_L_getula_Colubrinae	30.59	0.994	6.52	35.68
TJCS152_S_miliarius_Viperidae	21.60	0.994	7.21	30.09
TJCS631_D_margaritiferus_Colubrinae	32.43	0.994	7.85	36.47
TJCS146_S_miliarius_Viperidae	21.19	0.994	8.57	28.57
TJCS1221_I_cenchoa_Dipsadinae	22.54	0.994	4.85	29.63
TJCS1000_T_sartorii_Dipsadinae	46.11	0.994	3.32	50.96
TJCS1029_D_melanurus_Colubrinae	35.75	0.993	12.68	42.67
TJCS157RT_I_cenchoa_Dipsadinae	43.90	0.993	15.80	50.65
TJCS160_O_aestivus_Colubrinae	15.76	0.993	2.93	29.37
KW2028_P_obsoletus_Colubrinae	38.75	0.993	3.99	43.87

TJCS159_M_flagellum_Colubrinae	17.02	0.993	1.87	28.27
TJCS149RT_P_joberti_Dipsadinae	43.60	0.993	4.92	50.58
TJCS647b_L_mexicanus_Colubrinae	28.91	0.993	3.49	34.81
TJCS191_S_miliarius_Viperidae	24.62	0.993	4.61	33.28
TJCS820_C_imperalis_Dipsadinae	27.77	0.993	6.56	37.77
TJCS153RT_P_olfersii_Dipsadinae	53.05	0.993	23.87	59.09
TJCS1157_F_abacura_Dipsadinae	20.33	0.992	1.96	33.18
TJCS1018_L_frenata_Dipsadinae	18.21	0.992	2.38	30.48
KW2060_L_extenuata_Colubrinae	25.93	0.992	3.86	35.49
KW2011_R_rigida_Natricinae	26.50	0.992	4.71	38.52
TJCS25RTb_P_patagoniensis_Dipsadinae	83.74	0.992	34.78	89.69
TJCS163_T_sauritus_Natricinae	20.23	0.992	3.43	36.72
TJCS760_M_diastema_Elapidae	19.01	0.992	1.26	33.44
TJCS147_S_miliarius_Viperidae	25.51	0.992	3.13	35.29
TJCS24RTb_S_mikanii_Dipsadinae	72.18	0.992	29.13	79.40
TJCS1007_D_brevifacies_Dipsadinae	24.26	0.991	1.29	35.48
TJCS1213_L_septentrionalis_Dipsadinae	28.72	0.991	6.61	42.78
TJCS131RT_P_olfersii_Dipsadinae	34.44	0.991	1.91	48.51
TJCS168_D_puntatus_Dipsadinae	58.63	0.991	12.75	66.93
TJCS1216_B_asper_Viperidae	23.57	0.991	4.94	41.22
TJCS166_C_constrictor_Colubrinae	28.58	0.991	5.16	43.39
TJCS1209_B_asper_Viperidae	37.74	0.990	4.90	51.25
TJCS182_P_guttatus_Colubrinae	18.70	0.990	1.25	43.76
KW2159b_N_taxisilota_Natricinae	27.36	0.990	2.44	35.54
KW2045b_T_sauritus_Natricinae	34.94	0.990	3.82	49.52
TJCS61RT_T_melanocephala_Colubrinae	71.53	0.990	10.15	80.06
TJCS753_M_diastema_Elapidae	17.44	0.990	1.16	40.14
TJCS1192_X_rabdocephalus_Dipsadinae	50.45	0.990	11.58	58.28
TJCS144RT_E_poecilogyrus_Dipsadinae	38.89	0.990	1.56	47.30
TJCS190_T_sirtalis_Natricinae	27.11	0.989	3.64	48.56
TJCS1214_L_septentrionalis_Dipsadinae	35.94	0.989	10.55	54.13
TJCS186_T_sirtalis_Natricinae	31.30	0.989	2.99	48.86
TJCS34RTB_T_melanocephala_Colubrinae	53.70	0.989	17.18	69.79
TJCS183_M_fulvis_Elapidae	22.77	0.989	3.08	45.16
TJCs11RT_T_melanocephala_Colubrinae	92.36	0.989	21.84	101.28
TJCS137RT_M_aff_ibiboboca_Elapidae	26.52	0.989	4.49	47.31
KW1999b_N_fasciata_Natricinae	22.95	0.988	2.43	49.96
TJCS132RT_M_aff_ibiboboca_Elapidae	27.90	0.988	1.41	41.66
TJCS23RT_B_leucurus_Viperidae	27.76	0.988	6.79	58.61
TJCS139RT_Chironius_sp_Colubrinae	71.46	0.988	17.78	81.94
KW2157_L_getula_Colubrinae	53.57	0.988	13.98	67.03
TJCS51RT_A_paucisquamus_Typhlopidae	84.47	0.988	14.52	94.84
TJCS171_A_piscivorus_Viperidae	36.82	0.988	5.15	63.64

TJCS630_C_imperalis_Dipsadinae	31.00	0.988	6.80	57.00
TJCS161RT_M_aff_ibiboboca_Elapidae	23.55	0.988	2.61	51.39
TJCS170_C_constrictor_Colubrinae	38.99	0.988	10.91	61.57
TJCS72RT_E_poecilogyrus_Dipsadinae	80.78	0.988	5.21	89.05
KW2038_N_clarkii_Natricinae	34.67	0.987	7.98	54.32
TJCS113RT_A_brongersmianus_Typhlopidae	62.07	0.987	7.52	75.24
TJCS50RT_M_ibiboboca_Elapidae	72.26	0.987	6.89	82.67
TJCS167_C_constrictor_Colubrinae	72.73	0.987	16.46	83.62
TJCS666b_T_fasciata_Dipsadinae	36.39	0.987	5.34	50.94
TJCS105RTb_P_olfersii_Dipsadinae	80.21	0.987	24.22	95.11
KW2040_N_clarkii_Natricinae	45.77	0.987	8.21	59.12
TJCS2RT_E_almadensis_Dipsadinae	23.43	0.987	3.73	60.96
TJCS124RT_A_brongersmianus_Typhlopidae	47.35	0.987	5.94	61.77
TJCS122RT_E_taeniogaster_Dipsadinae	32.73	0.987	3.64	50.55
TJCS178_A_piscivorus_Viperidae	43.00	0.987	7.90	64.97
TJCS99RT_M_lemniscatus_Elapidae	41.53	0.987	3.29	57.51
TJCS1208_N_diademata_Dipsadinae	50.02	0.986	10.41	70.80
TJCS822_L_triangulum_Colubrinae	28.18	0.986	3.18	53.96
TJCS629b_C_imperalis_Dipsadinae	36.70	0.986	3.08	50.22
KW2051_C_coccinaea_Colubrinae	50.09	0.986	7.57	63.65
TJCS79RT_C_flavolineatus_Colubrinae	25.20	0.986	1.48	51.41
TJCS57RT_O_aeneus_Colubrinae	71.10	0.986	7.94	81.53
TJCS47RT_P_nattereri_Dipsadinae	29.54	0.986	2.77	53.59
TJCS4RT_B_lutzi_Viperidae	33.33	0.986	5.00	56.59
TJCS89RT_M_ibiboboca_Elapidae	61.61	0.986	3.77	76.88
TJCS96RT_M_ibiboboca_Elapidae	29.32	0.986	2.33	56.84
TJCS92RTb_S_mikanii_Dipsadinae	27.20	0.985	2.59	54.82
TJCS46RT_P_patagoniensis_Dipsadinae	78.95	0.985	7.28	91.42
TJCS181_A_contortrix_Viperidae	43.94	0.985	4.02	72.66
TJCS1188_X_rabdocephalus_Dipsadinae	39.90	0.984	6.61	76.69
TJCS91RT_P_patagoniensis_Dipsadinae	32.13	0.984	1.92	54.43
KW2050b_R_rigida_Natricinae	27.42	0.983	2.35	69.00
TJCS87RT_I_cenchoa_Dipsadinae	39.33	0.983	1.29	56.41
TJCS74RT_E_assisi_Boidae	40.84	0.983	3.46	74.94
TJCS135RT_M_lemniscatus_Elapidae	45.74	0.983	1.39	62.42
TJCS64RT_B_leucurus_Viperidae	43.21	0.983	2.57	67.19
TJCS129RTb_A_brongersmianus_Typhlopidae	99.70	0.982	18.81	113.90
TJCS95RT_C_flavolineatus_Colubrinae	43.23	0.982	3.71	77.32
TJCS613_C_schmidti_Dipsadinae	51.49	0.982	6.35	75.88
TJCS112RT_C_flavolineatus_Colubrinae	57.67	0.982	5.51	80.42
TJCS120RT_X_merremi_Dipsadinae	56.02	0.982	3.47	75.95
TJCS173_A_piscivorus_Viperidae	45.18	0.981	1.47	68.85
TJCS180_A_contortrix_Viperidae	67.28	0.981	5.61	81.86

TJCS63RT_O_trigeminus_Dipsadinae	49.31	0.981	2.03	71.01
TJCS119RT_P_nigra_Dipsadinae	78.97	0.980	13.40	99.92
TJCS138RT_Micrurus_sp_Elapidae	64.57	0.980	9.60	89.64
TJCS764_F_publia_Colubrina	66.66	0.980	5.17	86.58
TJCS78RT_P_nigra_Dipsadinae	85.68	0.979	7.82	103.92
TJCS76RT_H_angulatus_Dipsadinae	54.92	0.979	8.25	93.82
TJCS929_D_melanurus_Colubrinae	52.33	0.979	4.37	78.69
TJCS101RT_S_mikanii_Dipsadinae	47.80	0.979	1.97	85.25
TJCS68RT_L_dilepis_Dipsadinae	67.12	0.979	3.19	86.10
TJCS142RT_B_leucurus_Viperidae	36.67	0.978	1.73	85.15
TJCS81RT_E_assisi_Boidae	39.56	0.978	1.92	76.87
TJCS785_C_schmidti_Dipsadinae	60.82	0.978	6.39	89.27
TJCS60RT_A_paucisquamus_Typhlopidae	63.50	0.978	7.57	85.86
TJCS27RT_A_brongersmianus_Typhlopidae	65.90	0.978	6.17	96.01
TJCS162RT_Micrurus_sp_Elapidae	48.24	0.978	1.67	77.59
TJCS116RT_E_taeniogaster_Dipsadinae	69.88	0.978	4.45	105.32
TJCS928_B_constrictor_Boidae	50.17	0.977	3.16	90.33
TJCS70RT_A_paucisquamus_Typhlopidae	55.69	0.977	2.32	80.90
TJCS13RT_H_angulatus_Dipsadinae	41.73	0.977	1.33	84.32
TJCS24RT_S_mikanii_Dipsadinae	102.33	0.977	15.47	118.48
TJCS88RT_P_nigra_Dipsadinae	81.26	0.977	27.66	122.59
TJCS6RT_M_lemniscatus_Elapidae	45.74	0.977	1.48	82.83
TJCS784_L_frenata_Dipsadinae	68.96	0.976	13.10	107.39
TJCS12RT_H_angulatus_Dipsadinae	54.89	0.976	2.63	102.84
KW2003b_P_guttatus_Colubrinae	48.73	0.976	2.87	90.50
TJCS999_N_sebae_Dipsadinae	68.04	0.975	7.87	104.58
TJCS8RT_T_affinus_Dipsadinae	81.49	0.975	4.93	102.36
TJCS30RT_B_leucurus_Viperidae	112.65	0.975	13.49	131.24
TJCS630b_C_imperalis_Dipsadinae	56.39	0.974	4.06	106.51
TJCS152RTb_O_trigeminus_Dipsadinae	49.83	0.974	2.48	94.67
TJCS83RT_O_trigeminus_Dipsadinae	51.09	0.974	2.48	93.61
TJCS21RT_M_aff_ibiboboca_Elapidae	46.99	0.974	1.76	101.91
TJCS37RT_B_leucurus_Viperidae	51.47	0.974	1.74	96.55
TJCS34RT_T_melanocephala_Colubrinae	85.63	0.974	9.38	106.66
TJCS25RT_P_patagoniensis_Dipsadinae	113.53	0.974	15.50	132.62
KW2008b_N_fasciata_Natricinae	60.28	0.974	2.90	96.76
TJCS127RT_Chironius_RT528_Colubrinae	53.65	0.973	2.77	100.47
TJCS617b_D_margaritiferus_Colubrinae	57.41	0.973	4.27	101.62
TJCS643b_L_frenata_Dipsadinae	51.48	0.973	5.40	107.70
KW2039b_N_clarkii_Natricinae	59.96	0.972	2.84	99.55
TJCS106RTb_S_mikanii_Dipsadinae	56.30	0.972	2.65	104.30
TJCS117RT_P_nattereri_Dipsadinae	97.30	0.972	26.11	124.42
TJCS9RT_L_ahaetulla_Colubrinae	108.01	0.972	15.31	129.37

KW2026a_P_obsoletus_Colubrinae	57.76	0.972	2.67	102.47
TJCS1026KWb_L_getula_Colubrinae	53.87	0.972	2.52	103.48
KW2002b_T_sauritus_Natricinae	53.50	0.972	2.35	102.98
KW2041b_N_clarkii_Natricinae	64.03	0.972	3.53	113.96
TJCS1010_C_schmidti_Dipsadinae	59.89	0.971	2.24	98.88
TJCS28RT_E_poecilogyrus_Dipsadinae	80.83	0.971	11.37	126.69
KW1997b_T_sauritus_Natricinae	56.84	0.971	2.74	103.78
TJCS147RTb_E_poecilogyrus_Dipsadinae	57.63	0.971	2.72	111.80
KW2158b_H_platyrhinos_Dipsadinae	54.91	0.971	2.85	104.42
TJCS42RT_H_angulatus_Dipsadinae	63.32	0.971	10.23	122.14
TJCS115RT_D_dichrous_Colubrinae	94.93	0.971	12.19	124.32
KW1998b_T_sirtalis_Natricinae	55.83	0.970	2.55	106.86
TJCS15RT_T_melanocephala_Colubrinae	92.16	0.970	20.91	131.57
TJCS937_D_brevifacies_Dipsadinae	77.41	0.970	8.33	115.47
TJCS107RTb_P_olfersii_Dipsadinae	60.76	0.970	2.61	111.39
TJCS102RTb_P_nigra_Dipsadinae	61.16	0.970	2.94	111.29
KW2009a_N_fasciata_Natricinae	69.40	0.969	4.02	115.73
TJCS26RT_Chironius_sp_Colubrinae	57.72	0.969	2.11	109.59
TJCS29RT_S_nebulatus_Dipsadinae	106.11	0.969	5.49	132.12
TJCS5RT_M_ibiboboca_Elapidae	63.25	0.969	2.11	114.38
TJCS123RTb_H_angulatus_Dipsadinae	65.10	0.968	2.94	116.59
TJCS97RT_M_lemniscatus_Elapidae	50.56	0.968	3.12	132.61
TJCS104RTb_O_trigeminus_Dipsadinae	63.96	0.968	2.87	118.32
KW2027b_P_obsoletus_Colubrinae	68.19	0.967	3.29	118.48
TJCS1028KWb_N_floridana_Natricinae	69.00	0.967	4.03	121.98
TJCS35RT_L_ahaetulla_Colubrinae	68.99	0.967	1.87	109.34
TJCS158RTb_E_poecilogyrus_Dipsadinae	71.72	0.967	4.36	128.06
TJCS7RT_T_pallidus_Dipsadinae	107.99	0.967	5.81	134.99
TJCS80RT_P_patagoniensis_Dipsadinae	116.87	0.967	26.88	152.50
TJCS40RT_E_taeniogaster_Dipsadinae	69.44	0.966	6.24	134.27
KW1995b_N_fasciata_Natricinae	71.79	0.966	3.85	127.23
TJCS660b_D_margaritiferus_Colubrinae	77.56	0.965	4.43	125.75
TJCS66RT_P_olfersii_Dipsadinae	124.59	0.965	16.96	158.09
TJCS18RT_A_paucisquamus_Typhlopidae	89.86	0.964	19.79	147.01
TJCS126RTb_A_brongersmianus_Typhlopidae	129.12	0.962	15.38	165.12
TJCS39RT_X_merremi_Dipsadinae	95.32	0.961	9.44	154.69
TJCS16RT_A_paucisquamus_Typhlopidae	112.22	0.959	5.33	150.46
TJCS827_N_sebae_Dipsadinae	86.59	0.959	9.42	179.83
TJCS19RT_P_nattereri_Dipsadinae	96.12	0.957	4.05	157.44
KW2160b_N_fasciata_Natricinae	88.71	0.957	4.41	164.32
TJCS17RT_O_trigeminus_Dipsadinae	88.35	0.955	4.38	170.35
TJCS41RT_E_taeniogaster_Dipsadinae	122.60	0.951	13.79	178.64
TJCS3RT_E_assisi_Boidae	119.53	0.950	15.40	181.96

TJCS38RT_O_petolarius_Dipsadinae	94.43	0.948	10.38	196.02
TJCS22RT_B_constrictor_Boidae	107.02	0.942	2.75	216.69
TJCS36RT_S_nebulatus_Dipsadinae	195.53	0.938	40.78	256.43
TJCS1RT_L_dilepis_Dipsadinae	120.46	0.936	3.41	213.98

EDUCATION

2010   **M.Sc.**, Zoology (Major Professor: Dr. Laurie Vitt)

University of Oklahoma, Norman, Ok 71019

Thesis: Origins of Neotropical Diversity: Lineage Diversification in Tree Boas

2008   **B.S**., Zoology, University of Oklahoma, Norman, Ok 71019

ACADEMIC POSITIONS

2012 – 2015 **Research Affiliate,** Zoological Natural History Museum, Addis Ababa, Ethiopia.

2011 – 2012 **Instructor,** Northwest Community College, Oxford, MS.

RECENT GRANTS

2015	NSF Doctoral Dissertation Improvement Grant (DEB 1501711)	$18,419.00
2015	Society for Integrative & Comparative Biology, GIAR	$820.00
2014	Graduate Student Summer Research Grant Graduate College, University of Mississippi.	$3,000.00
2013	Graduate Student Council Research Grant University of Mississippi	$944. 00

2013	Linnean Society Grants in Systematics	£1140 ($1,857.00)
2013	Percy Sladen Memorial Fund	£600 ($907.00)
2013	Graduate Student Summer Research Grant Graduate College, University of Mississippi.	$3,000.00
2012	J. William Fulbright Student Fellowship	$26,009.60
2012	Graduate Student Summer Research Grant Graduate College, University of Mississippi.	$3,000.00
2012	T.H. Roosevelt Memorial Grant, American Museum of Natural History.	$2,000.00
2011	Graduate Student Summer Research Grant, Graduate College, University of Mississippi.	$2,600.00
2011	Orianne Society Student Travel Grant	$100.00

RECENT AWARDS AND HONORS

2016	U.M. Graduate School Dissertation Fellowship Award	$16,787.00
2015	SSAR Student Travel Award	$500.00
2014	University of Mississippi 3 Minute Thesis	*on campus finalist*
2011	American Society of Naturalist's Student Travel Award	$500.00

PUBLICATIONS

14. **Colston, T. J.** Book Review: Natural History of Neotropical Tree Boas (Genus *Corallus*).

—Robert W. Henderson (accepted in *The Quarterly Review of Biology).*

13. **Colston, T. J. (2017)** Gut microbiome transmission in lizards. *Molecular Ecology* 26: 972-974.

12. Ceriaco, L. M. P., Gutierrez, E. E., Dubois, A., ᵀ**Colston, T. J.,** *et al.* (2016) Photography-based taxonomy is inadequate, unnecessary, and potentially harmful for biological sciences. *Zootaxa* 4196(3):435-445. ᵀsupporting co-author/signatory.

11. **Colston, T. J.,** & Jackson, C. R. (2016) Invited Review: microbiome evolution along divergent branches of the vertebrate tree of life: what's known and unknown. *Molecular Ecology* 25: 3776-3800.

10. Gower, D.J., Wade, E.O.Z., Spawls, S., Böhme, W., Buechley, E., Siykes, D., **Colston, T.J.** (2016) A new large species of *Bitis* Gray, 1842 (Serpentes: Viperidae) from the Bale Mountains of Ethiopia. *Zootaxa* 4093(1):041-063.

9. Medina, M.F., Greenbaum, E., Bauer, A., Branch, W., Schmitz, A., Conradie, W., Nagy, Z. T., Hibbitts, T.J., Ernst, R., Portik, D.M., Nielsen, S.V., Kusamba, C., **Colston, T.J.,** Behangana, M. (2016) Phylogeny and systematics of *Panaspis* and *Afroablepharus* skinks (Squamata: Scincidae) in the savannas of sub-Saharan Africa. *Molecular Phylogenetics and Evolution* 100: 409-423.

8. **Colston, T. J.,** Barão-Nóbrega, J.A.L., Manders, R., Lett, A., *Wilmott, J., Cameron, G., *Hunter, S., Radage, A., Littlefair, E., Williams, R.J., Lopez Cen, A., Slater, K. (2015) Amphibians and reptiles of the Calakmul Biosphere Reserve, México with new records. *Check List* 11(5): 1759. * – **undergraduate researcher**

7. **Colston, T. J.,** Noonan, B. P., Jackson, C.R. (2015) Phylogenetic analysis of bacterial communities in different regions of the gastrointestinal tract of *Agkistrodon piscivorus*, the cottonmouth snake. *PloS one* 10.6: e0128793.

6. Nielsen, S. V. & **Colston, T. J.** (2014) The phylogenetic position of Ethiopia's sole endemic and biogeographically enigmatic cordylid lizard, *Cordylus rivae* (Squamata: Cordylidae), and a discussion of its conservation status. *African Journal of Herpetology* 63(2): 166-176.

5. Henderson, R. W., Pauers, M. J., **Colston, T. J.** (2013) On the congruence of morphology, trophic ecology, and phylogeny in Neotropical treeboas (Squamata: Boidae: *Corallus*). *Biological Journal of the Linnean Society*, 109: 466-475.

4. **Colston, T. J.,** Grazziotin, F.G., Shepard, D. B., Vitt, L. J., Coli, G. R., Henderson, R. W., Hedges, S. B., Bonatto, S., Zaher, H., Noonan, B.P., Burbrink, F. T. (2013) Molecular systematics and historical biogeography of tree boas (Genus *Corallus*)**.** *Molecular Phylogenetics & Evolution,* 66(3): 953-959.

3. Schlupp, I., **Colston, T. J.,** Joachim, B., Riesch, R. (2013) Translocation of cave fish (*Poecilia mexicana)* within and between natural habitats. *Environmental Biology of Fishes,* 22(2): 228-233.

2. Riesch, R., **Colston, T. J.,** Joachim, B. L., Schlupp, I. (2011) Natural history and life history of the Grijalva gambusia *Heterophallus milleri* Radda, 1987 (Teleostei: Poeciliidae). *Aqua*, 17(2): 95-102.

1. **Colston, T. J.,** Costa, G. C., Vitt, L. J. (2010) Snake diets and the Deep History Hypothesis. *Biological Journal of the Linnean Society,* 101: 476-486. –**undergraduate thesis**

TEACHING

Fall 2016 – **Microbiology,** Graduate Teaching Assistant, Department of Biology, University of Mississippi, Oxford, MS.

February 2016 – **Evolution,** <u>Guest Lecturer</u>, Department of Biology, University of Mississippi, Oxford, MS.

Spring 2015 – **Comparative Vertebrate Anatomy,** <u>Graduate Teaching Assistant</u>, Department of Biology, University of Mississippi, Oxford, MS.

January 2014 – **Biology of Invasive Species.** <u>Graduate Teaching Assistant</u>, Department of Biology, University of Mississippi, Oxford, MS.

Fall 2011 & Spring 2012 **Human Anatomy & Physiology I & II,** <u>Instructor</u>, Northwest Community College, Oxford, MS.

Fall 2011 & Spring 2012 **Introductory Biology I & II,** <u>Instructor</u>, Northwest Community College, Oxford, MS.

Summer 2011 **Comparative Vertebrate Anatomy,** <u>Graduate Teaching Assistant</u>, Department of Biology, University of Mississippi, Oxford, MS.

Spring 2011 **Comparative Vertebrate Anatomy,** <u>Graduate Teaching Assistant</u>, Department of Biology, University of Mississippi, Oxford, MS.

Fall 2010 **Comparative Vertebrate Anatomy,** <u>Graduate Teaching Assistant</u>, Department of Biology, University of Mississippi, Oxford, MS.

Summer 2010 **Field Herpetology,** <u>Instructor</u>, Sam Noble Oklahoma Museum of Natural History, Teaching Teachers Programs in Science in collaboration with Norman Public Schools.

Summer 2009 **Human Physiology,** <u>Graduate Teaching Assistant</u>, Department of Zoology, University of Oklahoma, Norman, OK.

Fall 2008 & Spring 2009 **Human Anatomy (cadaver course),** <u>Graduate Teaching Assistant</u>, Department of Zoology, University of Oklahoma, Norman, OK.

Spring 2009 **Parasitology,** <u>Graduate Teaching Assistant</u>, Department of Zoology, University of Oklahoma, Norman, OK.

Fall 2007 **Herpetology,** <u>Undergraduate Teaching Assistant</u>, Department of Zoology, University of Oklahoma, Norman, OK.

OTHER EXPERINCE

April 2014 – present **Contributing Personnel, Gene flow and riverine barriers in the Guiana Shield: a multitaxon test across the Oyapock River (MUsTARd). PI: Dr. Antoine Fouquet.** – Assist in generating and analyzing RAD-seq data for a CEBA funded project investigating the role of rivers as barriers to gene flow in the Guiana Shield (CEBA, ref. ANR-10-LABX-25-01, **19,800€**).

August 2011 – December 2014 **Graduate Research Assistant, University of Mississippi, Biology Department. Supervisor: Dr. Brice Noonan** – Research assistant on NSF funded project investigating patterns of diversification and biogeography of Malagasy ants using NGS technologies. (award #1120867).

COLLECTIONS EXPERIENCE

December 2016 – present **Graduate Curatorial Research Assistant,** University of Mississippi, Department of Biology Herbarium. – Responsible for catalog quality control and geo-referencing botanical collections.

August 2015 – December 2015 **Graduate Curatorial Research Assistant,** University of Mississippi, Department of Biology Museum Collections – Supervised specimen preparation of Ornithology, Herpetology and Mammalogy museum specimens.

October 2012 – December 2015 **Research Affiliate,** Department of Zoology and Museum of

Natural History, Addis Ababa University, Arat Kilo, Addis Ababa, Ethiopia.

August 1, 2009 – July 31, 2010 **Graduate Research Assistant,** Sam Noble Oklahoma Museum

of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan

Caldwell and Laurie Vitt – Herpetology collection research assistant. Assist in day-to-day

activities Including specimen preparation, maintenance, identification and loan requests.

Conduct collections-based research.

January 2008 – May 2008 **Graduate Curatorial Assistant,** Division of Herpetology, Sam Noble

Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK.

Supervisor: Dr. Laurie Vitt.

February 2006 – December 2007 **Undergraduate Collections Volunteer,** Division of

Herpetology, Sam Noble Oklahoma Museum of Natural History, University of

Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt.

PROFESSIONAL SERVICE

Peer Reviewer (No. Manuscripts) for *Acta Ethologica* (1)*, Biological Journal of the*

Linnean Society (3)*,* BMC *Evolutionary Biology* (1)*, Check List* (2)*, Herpetological*

Review (1)*, Journal of Zoo Biology* (1)*, Molecular Ecology* (4)*, Molecular Phylogenetics*

and Evolution (1)*, South American Journal of Herpetology* (1) *& The Southwestern*

Naturalist (1)

2017–present Member of the IUCN Boa and Python Specialist Group.

2016 Panel Member, SSAR graduate student workshop on grant writing and funding

opportunities. Joint Meetings of Ichthyologists & Herpetologists, New Orleans, LA,

USA.

2015–2016 University of Mississippi BGSA student representative (*faculty liaison*)

2015–2016 University of Mississippi BGSA Treasurer

2015–2016 Reviewer for Herpetologists' League EE Williams Research Grant

(Morphology/Systematics)

2014–present Contributor to IUCN's Chameleon Specialist Group, Horn of Africa.

2014–2016 University of Mississippi Graduate Student Council Representative (*Biology*

Senator)

2012–2016 Member of the University of Mississippi IACUC panel responsible for consultation

and training of field and laboratory methods pertaining to handling venomous reptiles.

2011, 2013, 2015 & 2017 External Grant Reviewer for Conservation Leadership Programme:

Partnership between Birdlife International, Conservation International, Fauna & Flora

International and Wildlife Conservation Society.

2010 Session Moderator (Biogeography) 2010 Joint Annual Meeting of the American Society

of Naturalists, Society of the Study of Evolution, and the Society of Systematic

Biologists Portland, Oregon, USA.

2010 University of Oklahoma ZAGS student representative (*faculty liaison*)

2010 University of Oklahoma ZAGS student representative (*graduate selections committee*)

SYNERGISTIC ACTIVITIES

International Collaborations: International collaboration is the foundation for much of my research program. Beginning in my undergraduate studies and continued throughout my graduate training I have established international collaborations with researchers and universities in Brazil, Ethiopia, French Guiana, Guyana, Mexico and the United Kingdom. Much of my research has included biogeographic studies of Neotropical herpetofauna and I have more recently laid the foundation for international collaborative work focusing on the biogeography of herpetofauna in the Horn of Africa.

Involving Undergraduates in Research: I have mentored several undergraduate researchers in the Noonan Lab and one of my mentees has gone on to a Ph.D. program in Ecology, Evolution and Organismal Biology. Ms. Megan Smith (Barksdale Honors Student and Taylor Medal awardee) gained extensive experience in DNA extraction, sequencing, and next generation library preparation and was awarded both a NSF REU and the prestigious NSF GRFP under my tutelage. Megan has moved on and has taken her GRFP with her to Bryan Carstens' lab at Ohio State University where she began her Ph.D. studies in the fall of 2015. Megan has been analyzing the data she generated on Ethiopian amphibian diversification patterns using samples I collected during my tenure as a Fulbright Student Scholar in Ethiopia, and has she has already presented at two international meetings (Evolution 2014, SSAR 2015). She has prepared one manuscript for submission based on these data and has a least one more that she will be the lead author on, yielding at least three publications from her undergraduate research under my tutelage.

My research role in Operation Wallacea's Mexico Project has involved supervision of 3 British undergraduate thesis students, and 2 of my thesis students have gone on to graduate studies and all 3 are in various stages of preparing their theses for publication in peer-reviewed journals. Undergraduate student volunteers are an integral part of my data collection in Brazil, Guyana, Ethiopia and Mexico.

PRESENTATIONS AT PROFESSIONAL MEETINGS

2016 **Colston T.J.,** Noonan, B.P., Jackson, C.R. The Evolution of Squamate Reptile Gut Microbiomes. Joint Meetings of Ichthyologists & Herpetologists, New Orleans, LA, USA.

2014 **Colston, T. J.,** Noonan, B.P., Jackson, C.R. Phylogenetic Analysis of Bacterial Communities in Different Regions of the Gastrointestinal Tract of *Agkistrodon piscivorus,* the Cottonmouth Snake. Biology of the Pitvipers 2, Tulsa, Ok, USA.

2013 **Colston, T. J.,** Jackson, C.R., Noonan, B.P. Snake Gut Microbial Communities: Ecological and Evolutionay Implications. Joint Meeting of the American Society of Naturalists, Society of the Study of Evolution, and the Society of Systematic Biologists, Snowbird, Utah, USA.

2010 **Colston, T. J.** Biogeography and Phylogeography of *Corallus hortulanus* with a review of the genus *Corallus*. Joint Annual Meeting of the American Society of Naturalists, Society of the Study of Evolution, and the Society of Systematic Biologists. Portland, Oregon, USA.

2010 **Colston, T. J.** Evolutionary Shifts in Dietary History of Snakes Impacts

Present Day Community Structure. III Annual Biology of Vipers Conference (**plenary**

lecuture). Pisa, Italy.

SEMINARS AND INVITED PUBLIC TALKS

2017 **Colston, T. J.** Evolution of the Squamate Reptile Gut Microbiome.

- LSU Museum of Natural Science Seminar Series.

2015 **Colston, T. J.** Uncovering the Herpetofauna of Abyssinia: Reptiles and Amphibians

in Ethiopia, the Land of People.

- Biology Seminar, University of Mississippi.

2015 **Colston, T. J.** Uncovering the Herpetofauna of Abyssinia: Reptiles and Amphibians

in Ethiopia, the Land of People.

- Austin Herpetological Society, Austin TX USA.

2014 **Colston, T. J.** Patterns and Processes of Community Assembly: an Investigation of New

World Snake Communities and Their Gut Bacteria.

- University of Mississippi Graduate College 3MT Competition *campus finalist

2012 **Colston, T. J.** Patterns and Processes of Community Assembly: An Investigation of New

and Old World Snake Communities and Their Gut Bacteria.

- Biology Seminar, University of Mississippi.

2012 **Colston, T. J.** Surveying the Serpent Kingdom.

- University of Mississippi Honors College.

2010 **Colston, T. J.** Origins of Neotropical Diversity: Lineage Diversification in Tree Boas.

- Museum Seminar, Sam Noble Museum & Zoology Department, University of Oklahoma.

2010 **Colston, T. J.** Origins of Neotropical Diversity: Lineage Diversification in Tree Boas.

- University of Oklahoma, Undergraduate Zoology Association.

2010 **Colston, T. J.** Origins of Neotropical Diversity: Lineage Diversification in Tree Boas.

- University of Oklahoma, Zoology Department eco-munch series.

2010 **Colston, T. J.** Evolutionary Shifts in Dietary History of Snakes Impacts Present Day Community Structure.

- III Annual Biology of Vipers Conference (**plenary lecuture**). Pisa, Italy

2010 **Colston, T. J.** Evolutionary Shifts in Dietary History of Snakes Impacts Present Day Community Structure.

- University of Oklahoma Darwinathon. Norman, OK.

PHOTOGRAPHY

My passion for field-based research has led me to develop a passion for documenting both my study organisms and the ecosystems in which they occur. Example images can be found on my Flickr site: http://www.flickr.com/photos/15023943@N05/ or through my personal webpage http://maddreptiles.com. Publications include:

- Herpetology: An Introductory Biology of Amphibians and Reptiles. 3rd & 4th eds. Vitt & Caldwell, Academic Press.

- Natural History of Neotropical Treeboas. Henderson, ECO Publishing.

- Calphotos, CITES, EMBL Reptile Database, Encyclopedia of Life (EOL), and IUCN contributor since 2010.