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# Comparison of the gut microbiomes of 12 bony fish and 3 shark species

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ABSTRACT: We used massively parallel sequencing (pyrosequencing) of 16S rRNA genes to compare the composition of microbial communities in the guts of 12 bony fish and 3 shark species. The species analyzed encompass herbivores and carnivores with varied digestive physiologies, are classified as pelagic and demersal species, and reside in estuarine to marine environments. We also compared the gut microbial assemblages of wild and cultured *Fundulus heteroclitus* and of juvenile and adult *Lagodon rhomboides*. A total of 1 214 355 sequences were filtered, denoised, trimmed, and then sorted into operational taxonomic units (OTUs) based on 97% sequence similarity. Bacteria representing 17 phyla were found among the sampled fish, with most fish hosting between 7 and 15 phyla. *Proteobacteria* OTUs were present in all fish and often dominated the libraries (3.0 to 98%; average: 61%). *Firmicutes* were also prevalent, but at a lower relative abundance, ranging between 1.3 and 45% (average: 17%). In most cases, the gut microflora of individual fish of a given species contained many of the same OTUs; however, some species (e.g. great barracuda) shared few OTUs among the individuals sampled. Although no single OTU was shared among all fish species, many of the OTUs present in one species' core group were also found in the core groups of other species. Several OTUs were consistently found in the guts of multiple species, suggesting that these OTUs may be important contributors to fish gut functions such as digestion, nutrient absorption, and immune response.

KEY WORDS: Fish gut · Gut microbiome · 16S rRNA · Gut microflora · 454-pyrosequencing · Shark gut · Core gut microbiome



## INTRODUCTION

Skin, gills, eggs, and intestinal tracts of fish all harbor abundant populations of bacteria (MacFarlane et al. 1986, Cahill 1990) that impact the overall health and physiology of the host. Fish intestines in particular harbor large and diverse populations of bacteria (Austin & Austin 1987, Cahill 1990, Ringø et al. 1995). Most studies have shown that this gut microflora varies among fish species, and that the dominant bacteria are typically either aerobes or facultative anaerobes (Ringø et al. 1995). However, some studies have documented obligate anaerobes as part of the gut microbial assemblage (Trust et al. 1979, Ringø et al. 1995). Izvekova et al. (2007) reviewed studies of fish gut microflora published between 1929 and 2006 and found that of the 73 bacterial taxa documented 53% were Gram-negative aerobes, 34% were Gram-positive aerobes, 8.2% were Gram-negative anaerobes, and 4.1% were Gram-positive anaerobes.

Direct comparisons between past studies are hampered by inconsistencies in the methods used. Studies conducted prior to ~2005 relied on culture-based techniques to enumerate and identify bacteria

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(Newman et al. 1972, MacFarlane et al. 1986, Spanggaard et al. 2000, Aschfalk & Müller 2002, Verner-Jeffreys et al. 2003, Al-Harbi & Naim Uddin 2004, Martin-Antonio et al. 2007, Skrodenytė-Arbačiauskienė 2007). These studies have provided valuable insights into the composition of microbial communities and have yielded isolates for detailed physiological investigation; however, they are likely to have provided biased assessments of the microbial community composition, as typically <1% of the cells known to be present by direct microscopic enumeration produce colonies on solid media (Ferguson et al. 1984, Head et al. 1998), which were formerly a crucial step in identifying bacteria. With that caveat, Table S1 (in the Supplement at www.int-res.com/articles/suppl/m518p209_supp.pdf) lists the dominant gut microflora reported in published studies of a variety of fresh- and saltwater fish species from wild and cultured populations. Most of these studies only examined a single fish species and used a variety of culture-dependent and culture-independent methodologies to assess microflora community composition.

Based on this review of the literature (Table S1), the gut microbiomes of most fish are dominated by γ-*Proteobacteria* such as *Aeromonas* sp., *Escherichia coli, Photobacterium* sp., *Pseudomonas* sp., and *Vibrio* sp. However, some fish such as the Atlantic salmon *Salmo salar* (Holben et al. 2002) and the long-jawed mudsucker *Gillichythys mirabilis* (Bano et al. 2007) have intestinal microflora dominated by *Tenericutes* (*Mycoplasma* sp.). Lactic acid bacteria (mainly *Lactobacillus* sp.) have also been found to be minor components of the gut microflora of both freshwater and marine fish (Izvekova et al. 2007). Unlike bony fish, there has been little research on the gut microbiomes of sharks. One culture-dependent study found that *Photobacterium damselae* was a normal member of their gut microflora (Grimes et al. 1985), but there have been no culture-independent analyses of the shark gut microbiome.

The gut microbial community can respond to a variety of factors affecting the host, including changing environmental conditions such as temperature and salinity (Yoshimizu & Kimura 1976, MacFarlane et al. 1986), developmental stage (Verner-Jeffreys et al. 2003, Romero & Navarrete 2006), digestive physiology (Cahill 1990), and feeding strategy (Uchii et al. 2006). Some of the gut microflora appear to be transient, while other bacteria seem to be permanent residents (Kim et al. 2007). Resident gut microflora are those bacteria from the diet or environment that are able to colonize, persist, and proliferate within the gut (Sugita et al. 1988, Cahill 1990). Within a species' natural habitat, stable environmental conditions may lead to the establishment of a stable gut microflora that is representative of the 'natural flora' of the species (Lynch & Hobbie 1988, Oxley et al. 2002). However in culture systems, conditions of diet, water quality, and population density may be very different from those of the natural habitat. This may result in differences between the gut microflora of wild and cultured populations of the same species, and indeed, MacFarlane et al. (1986) observed that farm-raised fish had a simpler gut flora than their wild counterparts.

Several studies have shown that many herbivorous fish such as the pinfish *Lagodon rhomboides* undergo an ontogenetic diet shift, transitioning from carnivorous juveniles to either omnivorous or herbivorous adults (Benavides et al. 1994, Muñoz & Ojeda 2000, Gallagher et al. 2001). Luczkovich & Stellwag (1993) indicated that this ontogenetic shift in diet resulted in both qualitative and quantitative variability in the composition of the *L. rhomboides* gut microflora. Considering the likely significance of gut microflora in digestion and nutrient acquisition, fish adapted to a carnivorous lifestyle likely have gut microbial assemblages that are different from those that feed on plant material.

We used massively parallel sequencing (pyrosequencing) of *Bacteria* 16S rRNA genes to test hypotheses about the relationship between gut microflora composition and lifestyle in 12 bony fish and 3 shark species, selected to encompass a wide range of lifestyles. The fish species sampled include both herbivores and carnivores, represent varied digestive physiologies, are classified as pelagic or demersal species, and reside in estuarine to marine environments. We also included 3 species of sharks that, unlike bony fish, have a short intestine incorporating a spiraled valve (Budker & Whitehead 1971) that increases the intestinal surface area and allows for increased absorption (Castro & Huber 2003).

## MATERIALS AND METHODS

### Fish collection

Table 1 lists the species used in this study, along with their phylogenetic classification, feeding strategies, common habitats, and digestive physiologies. In addition to the 15 fish species used for interspecific comparison, we also compared wild and cultured *Fundulus heteroclitus* (mummichogs) and juvenile

Table 1. Fish species sampled in this study, including their habitat (preferred environment and salinity range), overall feeding strategy (C: carnivore, H: herbivore, O: omnivore; based on Froese & Pauly 2011) with specific categories in brackets, and digestive physiology

| Common Name | ID | Species | Order | Family | Habitat | Feeding strategy | Digestive physiology |
|---|---|---|---|---|---|---|---|
| Mummichog | MC | *Fundulus heteroclitus* | Cyprinodonti-formes | Fundulidae | Benthopelagic; freshwater-marine | O | Simple tube |
| Pinfish | PF | *Lagodon rhomboides* | Perciformes | Sparidae | Demersal; brackish-marine | C/H[a] (herbivore, invertivore) | Differentiated, elongated intestine |
| Silver perch | SP | *Bairdiella chrysoura* | Perciformes | Sciaenidae | Demersal; brackish-marine | C (invertivore) | Differentiated |
| Black sea bass | BSB | *Centropristis striata* | Perciformes | Serranidae | Reef-associated; marine | C (invertivore, piscivore) | Differentiated |
| Hogchoker | HC | *Trinectes maculatus* | Pleuronectiformes | Achiridae | Demersal; freshwater-marine | O (herbivore, invertivore) | Short intestine, pyloric caeca |
| Southern flounder | FL | *Paralichthys lethostigma* | Pleuronectiformes | Paralichthyidae | Demersal; brackish-marine | C (piscivore) | Short intestine, pyloric caeca |
| Spanish mackerel | SPM | *Scomberomorus maculatus* | Perciformes | Scombridae | Pelagic-neritic; marine | C (piscivore) | Folded intestine, pyloric caeca |
| King mackerel | KM | *Scomberomorus cavalla* | Perciformes | Scombridae | Reef-associated; marine | C (piscivore) | Folded intestine, pyloric caeca |
| Red drum | RD | *Sciaeops ocellatus* | Perciformes | Sciaenidae | Demersal; brackish-marine | C (invertivore, piscivore) | Folded intestine, pyloric caeca |
| Crevalle jack | JC | *Caranx hippos* | Perciformes | Carangidae | Reef-associated; brackish-marine | C (invertivore, piscivore) | Pyloric caeca |
| Mahi-mahi | MH | *Coryphaena hippurus* | Perciformes | Coryphaenidae | Pelagic-neritic; brackish-marine | C (piscivore) | Short intestine, pyloric caeca |
| Great barracuda | BR | *Sphyraena barracuda* | Perciformes | Sphyraenidae | Reef-associated; brackish-marine | C (piscivore) | Short intestine |
| Striped burrfish | SB | *Chilomycterus schoepfi* | Tetraodontiformes | Diodontidae | Reef-associated; marine | C (invertivore) | Differentiated |
| Spinner shark | SPN | *Carcharhinus brevipinna* | Carcharhiniformes | Carcharhinidae | Reef-associated; marine | C (piscivore) | Short intestine, spiraled valve |
| Atlantic sharpnose shark | SHP | *Rhizoprionodon terraenovae* | Carcharhiniformes | Carcharhinidae | Demersal; brackish-marine | C (piscivore) | Short intestine, spiraled valve |
| Sandbar shark | SDB | *Carcharhinus plumbeus* | Carcharhiniformes | Carcharhinidae | Benthopelagic; brackish-marine | C (piscivore) | Short intestine, spiraled valve |

[a]*L. rhomboides* undergo an ontogenetic diet shift from carnivorous to herbivorous

and adult *Lagodon rhomboides* (pinfish). We sampled 4 mummichogs each from wild and cultured populations, 4 pinfish each from juvenile and adult populations, and 2 to 3 fish for all other species, using trap, trawl, or hook and line. Mummichogs, silver perch, and hogchokers were euthanized with tricaine methanesulfonate (MS-222; Sigma) within 1 h of capture. All other fish, except pinfish, were placed immediately on ice upon capture. Pinfish were euthanized with MS-222 after being held in recirculating tanks for no longer than 4 h after capture (pinfish were not fed during this period). Recirculating tanks were filled with water from the same environment where fish were caught.

Wild mummichog specimens were collected in the USA from Sapelo Island, GA, and cultured fish were collected from a population near Beaufort, NC, then reared in captivity for 11 generations at the Aquatic Biotechnology and Environmental Lab, University of Georgia (courtesy of Dr. R. Winn) before being used in these experiments. Cultured fish had been reared in recirculating seawater culture systems and fed a diet of brine shrimp (San Francisco Bay Brand), freeze-dried plankton (San Francisco Bay Brand), and Otohime EP1 (Pentair Aquatic Eco-Systems).

Pinfish were collected by trawl from the Gulf of Mexico (29° 52′ N, 84° 29′ W) with logistic support from the Florida State University Coastal & Marine Laboratory (St. Teresa, FL). Pinfish were classified as juveniles (<120 mm body length) or adults (>120 mm body length). All fish were kept in recirculating tanks for no longer than 4 h prior to dissection.

### Dissections and DNA extractions

The exterior of each fish was cleaned with 95% ethanol prior to dissection. Microbes attached to the intestinal wall were considered to be part of the natural gut microflora (Ringø et al. 2001), and thus, the whole intestine and not just lumen contents were used for all extractions.

The mid- to hind-gut region of the intestine was removed, sliced open, and placed in a PowerBead tube (MoBio Laboratories). The intestines of several species including southern flounder, black sea bass, red drum, crevalle jack, Spanish mackerel, king mackerel, mahi-mahi, great barracuda, spinner shark, Atlantic sharpnose shark, and sandbar shark were too large to fit directly into PowerBead tubes. These intestines were placed in 50 or 250 ml tubes with phosphate-buffered saline solution and sonicated for 30 min. The supernatant was decanted into another tube then centrifuged at 15008 × *g* for 5 min. The pellet was transferred directly into a PowerBead tube using a sterile spatula. DNA extractions were then completed using the MoBio Power Soil DNA Extraction Kit according to manufacturer's instructions.

### 16S rRNA pyrosequencing and analysis

We analyzed the distribution of 16S rRNA operational taxonomic units (OTUs) with massively parallel sequencing (pyrosequencing) using a Roche 454/FLX instrument running Titanium chemistry. Bacterial DNA was amplified using universal 16S rRNA primers 27F and 338R-I and -II (Roeselers et al. 2011), which were modified with Titanium (Lib-L) adaptors and sample-specific barcodes. PCR assays were performed in triplicate using Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific) and 1 µM forward and reverse (pooled 338R-I & -II) primers with the following conditions: initial denaturation at 95°C for 10 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min.

PCR products were pooled following amplification and purified using Agencourt Ampure XP (Beckman Coulter) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified (Quant-iT PicoGreen; Invitrogen), pooled in equal concentration and submitted to the Georgia Genomics Facility (University of Georgia, Athens, GA) for sequencing. A total of 1 214 355 sequences was obtained. These sequences were filtered, denoised using the denoise_wrapper.py script, checked for chimeras using ChimeraSlayer, then sorted into OTUs using UCLUST with a 0.97 similarity threshold, and aligned with PyNast using the UCLUST pairwise alignment method through the Qiime software pipeline (Caporaso et al. 2010, 2011). Taxonomy was assigned using the RDP classifier and the Greengenes reference database in Qime. Sequences have been submitted to the NCBI Short Read Archive under accession number SRP033284. All chloroplasts and unassigned sequences (defined as those not binned to the kingdom level) were removed from the data set before further analysis. OTUs shared by all of the fish sampled from a given species were defined as the core group of OTUs for that species. Rarefaction curves were determined using the alpha_rarefaction.py script in Qiime for the Chao1, Observed Species, Phylogenetic Diversity (PD) Whole Tree, and Shannon metrics. The alpha diver-

sity metrics provided estimates of the richness and diversity of the community. The Chao1 metric assessed species richness, Observed Species counted the number of unique OTUs found within a sample (richness), Phylogenetic Diversity incorporated branch lengths of taxa from a phylogenetic tree and measured phylogenetic diversity (Faith & Baker 2006), and the Shannon index estimated species diversity including both richness and evenness (Caporaso et al. 2010, 2011).

We used the jackknifed_beta_diversity.py workflow script in Qiime (Caporaso et al. 2010, 2011) to compare the gut microbiomes of individual fish. This analysis assessed the robustness of our sequencing effort (Caporaso et al. 2010, 2011) and determined how often individual microbiomes clustered randomly (Lozupone et al. 2011). The analysis used weighted UniFrac (based on normalized abundance data) distances from our complete OTU table at an even sampling depth for all samples. A consensus tree was constructed from 999 jackknifed iterations using UPGMA (unweighted pair group method with arithmetic mean) clustering. Additionally, we used the compare_categories.py workflow script with the method set to ANOSIM to determine if there were statistically significant differences among all OTUs from cultured and wild mummichogs, juvenile and adult pinfish, and the 12 bony fish and 3 shark species.

We also used the software package PRIMER (v.6; Clarke & Gorley 2006) for non-metric multidimensional scaling (NMDS) visualization of core OTUs in the gut microbiomes of each species. Core OTUs were transformed as presence/absence of individual OTUs shared among all samples of a species. The multi-response permutation procedure (MRPP) performed in R (R Core Team 2009) with the 'vegan' statistical package (Oksanen et al. 2009) was used to test whether there were significant differences between clustered groups of samples. MRPP was run with the Bray-Curtis distance matrix with 999 permutations. Additional statistical analyses, including *t*-test, Kruskal-Wallis 1-way ANOVA, and pairwise Wilcoxon rank sum tests were performed in R (R CoreTeam 2009) using the 'vegan' statistical package (Oksanen et al. 2009). For all tests, we considered a significance level of $\alpha = 0.05$.

### 16s rRNA Sanger sequencing and analysis of sequences from clone libraries

DNA from mummichog (n = 5), pinfish (n = 11), silver perch (n = 3), black sea bass (n = 4), striped burrfish *Chilomycterus schoepfi* (n = 4), Japanese medaka *Oryzius latipes* (n = 10 pooled fish), spinner shark (n = 2), and Atlantic sharpnose shark (n = 2) was also amplified using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with the *Bacteria*-specific 16S rRNA primers 27F and 1492R (Lane 1991) under the following PCR conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min; finishing with a final extension at 72°C for 45 min. Amplified DNA was electrophoresed on a 1% agarose gel, bands of the expected product size were excised, then the DNA they contained was extracted and purified using QIAGEN QIAquick gel extraction kits (Qiagen). DNA extracted from the gel was cloned with TOPO TA cloning kits (Invitrogen) using the pCR 4.0-TOPO TA vector and competent *E. coli* cells. Clones were selected randomly and sequenced using the 27F primer by Genewiz. All sequences were checked for chimeras using the Bellerophon server (Huber et al. 2004). Sequences were identified by both RDP SeqMatch (Cole et al. 2007, 2009) and by BLAST (Johnson et al. 2008) against the non-redundant nucleotide database (NCBI GenBank), and aligned using ClustalW (Larkin et al. 2007). Phylogenetic trees were constructed using MEGA 5.05 (Tamura et al. 2011). Sequences have been deposited in GenBank, accession numbers KJ197337 to KJ197858.

## RESULTS

### Cross species comparison

Pyrosequencing yielded a total of 1 214 355 sequences. A total of 1 038 277 sequences remained in the data set after filtering for quality and chimeras. Most samples contained 0 to 10% (average 2.3%) chloroplast sequences; however, the libraries of cultured mummichog specimens 2, 3, and 4 contained more (59, 96, and 67% respectively). A total of 719 216 sequences remained after removing sequences from chloroplasts and unassigned OTUs (Table S2 in the Supplement). These sequences were assigned to 2226 OTUs (97% similarity) binned to 16 phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Caldithrix*, *Chlorobi*, *Chloroflexi*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Thermi*, and *Verrucomicrobia*). OTUs sorted into the candidate phyla of OP11, SBR1093, TM6, TM7, WPS-2, WS3, and WS6 were combined into an 'other

phyla' category that contained between 0 and 3.7% (average 0.16%) of the sequences from each sample.

Not only were there differences among the different bony fish and sharks in terms of the bacterial phyla present in their guts, but there was also variability among individuals of the same species (Fig. 1). The within-species variability was more marked in some fish, and was particularly extreme for king mackerel and great barracuda. Despite this variability, representatives of the same bacterial phyla were found in the guts of all samples of individual fish species, though relative abundance varied. Excluding the category 'other phyla,' richness (at the phylum level) of the gut microbiomes of different fish species ranged from 7 to 15 phyla (average = 11; Fig. S1 in the Supplement). Microbiomes from the guts of red drum contained the richest microbial communities among the fish we sampled, whereas those from mahi-mahi and sandbar shark were the simplest. The phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* were found in all 15 fish gut microbiomes. The phyla *Spirochaetes* and *Tenericutes* were recovered from the guts of 13 of the fish species.

*Proteobacteria* dominated the gut microbiomes of most species, accounting for 3 to 98% (mean ± SD = 61 ± 34%) of the sequences retrieved. *Firmicutes* were found in all species, but at lower relative abundance (range = 1.3 – 45%, mean ± SD = 17 ± 22%) of the sequence library. Within the *Firmicutes*, *Lactobacillales* OTUs were found in all fish species except mahi-mahi. For most fish species, *Lactobacillales* contributed <1% of the sequences retrieved. However, *Lactobacillales* were more abundant among cultured mummichogs (2.2% of sequences), crevalle jack (2.1%), and Spanish mackerel (13%).

*Spirochaetes* contributed <1.1% of the sequences recovered from all species except for mahi-mahi and barracuda, where they accounted for 64 to 98% (83 ± 17%) and 0.05 to 99% (34 ± 57%), respectively, of the sequences we retrieved. *Tenericutes* accounted for 1.6, 7.9, 2.6, and 1.3% of all sequences retrieved from wild mummichogs, juvenile and adult pinfish, and crevalle jacks, respectively, averaged across all samples of a given fish species. *Tenericutes* contributed 18 and 82% of the sequences retrieved from the guts of 2 king mackerel specimens.

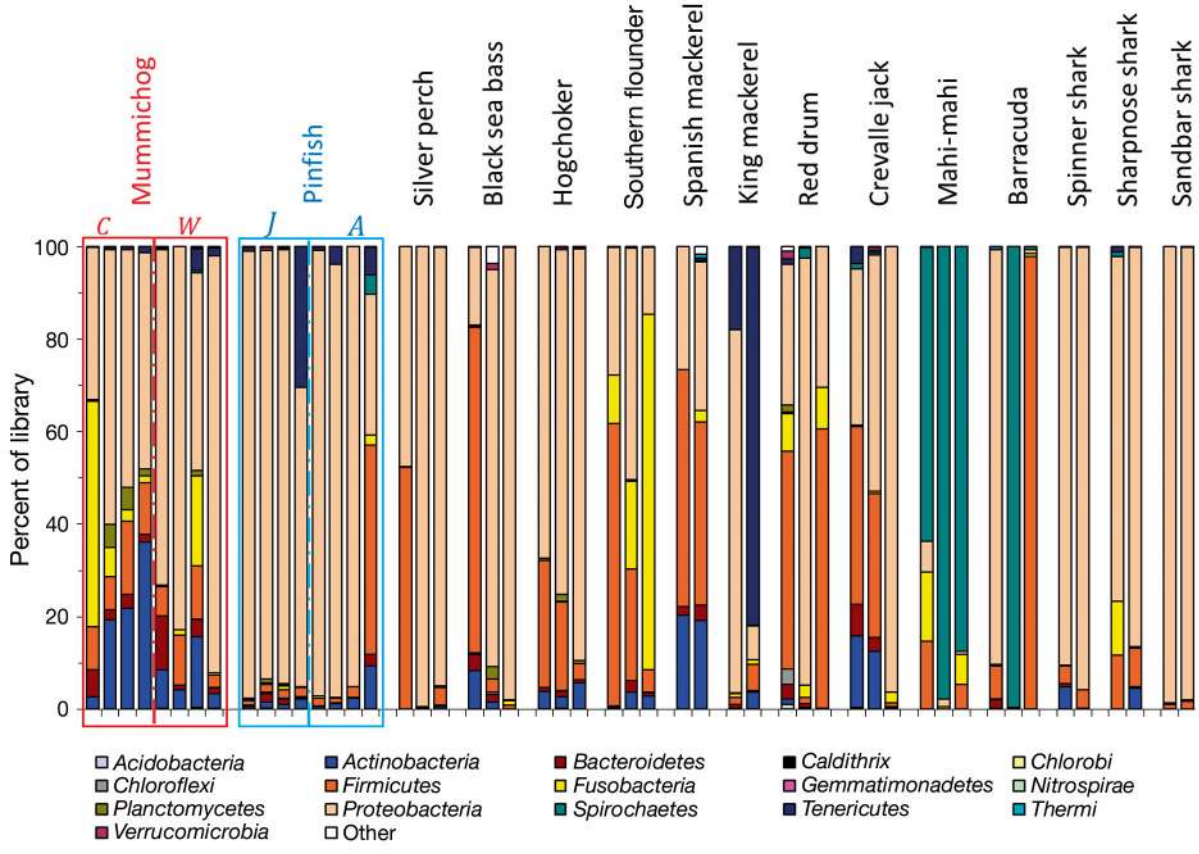


Fig. 1. Distribution of bacterial phyla (as % of operational taxonomic units retrieved) in individual samples of 12 bony fish and 3 shark species determined with 454-pyrosequencing. C: cultured population, W: wild population, J: juveniles, A: adults

Within each fish species, we found that the individual bony fish and sharks sampled shared 7 to 60 OTUs (Table 2). For the sake of simplicity we defined the OTUs shared by all of the fish sampled from a given species as the core OTU group for that species, recognizing that this simplification has greater validity for species that had several samples (i.e. mummichog and pinfish) versus those for which only 2 fish were sampled (e.g. Spanish mackerel) or for which the microbiomes from guts of individual fish were highly divergent (e.g. great barracuda). Many of the OTUs present in one species' core group were also present as members of the core groups of other species; however, no OTU was shared among all species. The 3 shark species shared a core microbiome containing 3 OTUs assigned to *Cetobacterium* sp., *Photobacterium* sp., and *Vibrio* sp. Core gut microbiomes of most bony fish and sharks contained OTUs from the phyla *Actinobacteria*, *Fusobacteria*, *Firmicutes*, and *Proteobacteria* (mainly γ-*Proteobacteria*).

OTUs assigned to the Family *Vibrionaceae* were present in the core group of all fish except Spanish mackerel. With the exception of mummichog and Spanish mackerel, the core groups of all species contained OTUs similar to *Photobacterium* sp. OTUs assigned to *Propionibacterium* sp., *Vibrio* sp., and *Pseudomonas* sp. were present in the core groups of 87, 67 and 67%, respectively, of all species sampled.

Table 2. Core gut microflora of species sampled. The core gut microflora was defined as the operational taxonomic units (OTUs) found in all samples of a species. The top 5 core OTUs are listed in order of greatest abundance. Numbers in parentheses following a taxon identifier indicate the number of different OTUs (if >1) recovered within that taxon. n = sample size

| Species | n | No. of shared OTUs | % of total shared sequences | Top 5 core OTUs (by abundance) |
|---|---|---|---|---|
| Cultured mummichog | 4 | 27 | 50–68 | *Cetobacterium* sp., *Propionibacterium* sp., *Vibrio* sp., *Acidovorax* sp., *Pseudomonas* sp. |
| Wild mummichog | 4 | 41 | 28–76 | *Vibrio* sp., *Photobacterium* sp., *Pseudomonas* sp., *Halomonas* sp., *Propionibacterium* sp. |
| Mummichog (all) | 8 | 12 | 7–58 | *Vibrio* sp., *Propionibacterium* sp., *Pseudomonas* sp., *Moraxellaceae*, *Acidovorax* sp. |
| Juvenile pinfish | 4 | 43 | 65–91 | *Vibrio* sp., *Enterovibrio* sp., *Vibrionaceae*, *Staphylococcus* sp., *Propionibacterium* sp. |
| Adult pinfish | 4 | 14 | 14–93 | *Shewanella* sp., *Halomonas* sp., *Photobacterium* sp., *Propionibacterium* sp., *Corynebacterium* sp. |
| Pinfish (all) | 8 | 10 | 1.1–14 | *Photobacterium* sp., *Propionibacterium* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Corynebacterium* sp. |
| Silver perch | 3 | 20 | 69–99 | *Photobacterium* sp. (2), *Clostridiaceae*, *Vibrionaceae* (2) |
| Black sea bass | 3 | 12 | 9–81 | *Photobacterium* sp., *Propionibacterium* sp., *Ruegeria* sp., *Corynebacterium* sp., *Escherichia* sp. |
| Hogchoker | 3 | 36 | 61–92 | *Shewanella* sp., *Halomonas* sp., *Propionibacterium* sp., *Pseudomonas* sp. (2) |
| Southern flounder | 3 | 21 | 12–41 | *Photobacterium* sp., *Clostridiaceae*, *Clostridium* sp., *Clostridiaceae* (2) |
| Spanish mackerel | 2 | 26 | 57–62 | *Alicyclobacillus* sp., *Propionibacterium* sp., *Pseudomonas* sp. (2), *Corynebacterium* sp. |
| King mackerel | 2 | 60 | 94–96 | *Photobacterium* sp., *Ureaplasma* sp., *Acinetobacter* sp., *Cetobacterium* sp., *Alicyclobacillus* sp. |
| Red drum | 3 | 15 | 16–74 | *Photobacterium* sp., *Cetobacterium* sp., *Clostridiaceae* (2), *Vibrio* sp. |
| Crevalle jack | 3 | 20 | 20–91 | *Photobacterium* sp., *Alicyclobacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Propionibacterium* sp. |
| Mahi-mahi | 3 | 13 | 98–99 | *Brachyspira* sp., *Spirochaetes*, *Ruminococcaceae*, *Cetobacterium* sp., *Photobacterium* sp. |
| Great barracuda | 3 | 7 | 0.10–74 | *Photobacterium* sp., *Acinetobacter* sp. (2), *Escherichia* sp., *Enterobacteriaceae* |
| Sharpnose shark | 2 | 19 | 69–74 | *Photobacterium* sp. (2), *Vibrio* sp., *Campylobacter* sp., *Propionibacterium* sp. |
| Spinner shark | 2 | 14 | 82–90 | *Photobacterium* sp., *Propiongenium* sp., *Clostridiaceae*, *Clostridium* sp., *Vibrio* sp. |
| Sandbar shark | 2 | 8 | 97–98 | *Photobacterium* sp. (2), *Vibrio* sp. 1, *Cetobacterium* sp., *Vibrio* sp. 2 |

OTUs assigned to *Escherichia* sp., *Staphylococcus* sp., *Streptococcus* sp., *Clostridiaceae*, *Clostridium* sp., *Acinetobacter* sp., *Corynebacterium* sp., *Cetobacterium* sp., *Shewanella* sp. were also recovered from many (40 to 53%; Table S3 in the Supplement) of the species sampled. The *Lactobacillales* OTUs assigned to *Lactobacillus* sp. and *Streptococcus* sp. were part of the core group of OTUs from mummichog, pinfish, hogchoker, southern flounder, Spanish mackerel, king mackerel, and crevalle jack.

Rarefaction curves for the Chao1, Observed Species, Phylogenetic Diversity (PD) Whole Tree, and Shannon alpha diversity metrics are shown in Fig. S2 in the Supplement. Table S4 lists the results of the 4 alpha diversity metrics for all samples. Table 3 compares these alpha diversity metrics across species. Gut microbiomes from wild mummichogs had the greatest richness, greatest phylogenetic diversity, and the second-most diverse gut microflora assemblages. Spanish mackerel had the highest diversity. Mahi-mahi, barracuda, and sandbar shark had the least complex assemblages as measured by richness, phylogenetic diversity, and diversity (Table 3). The silver perch gut microbiome was also less rich than most of the other bony fish gut microbiomes except for those of the mahi-mahi and barracuda. The gut microbiomes of all 3 shark species were less rich and diverse than most bony fish species (i.e. mummichog, pinfish, black sea bass, Spanish mackerel, and crevalle jack). Of the shark species, the gut microbiome of the sandbar shark had the lowest diversity, and spinner shark had the greatest diversity.

We compared the gut microflora communities from the fish we sampled using jackknifed analysis of weighted UniFrac distances (Fig. 2). The analysis indicated that all bony fish and shark samples, except barracuda specimen 2 (BR2), cluster together with >75% jackknifed support. Microbiomes from different fish of the same species did not always cluster with each other, reflecting within-species variability in gut microbiome composition. There is >75% support for the mahi-mahi and sandbar shark clusters that include all specimens of each species. The core groups of microflora for each fish species were also compared using NMDS to visualize groupings (Fig. 3). MRPP indicates that clusters defined at 20, 30, 40, and 50% similarity are significantly different ($p = 0.001$). This analysis showed that the core group from barracuda was markedly different from those of the other fish. The core groups of the remaining fish formed 2 clusters at >20% similarity. One cluster included mahi-mahi, red drum, silver perch, and the shark species. The second cluster included both mackerel species; however, there is little similarity between the core groups of the 2 mackerel species and they do not group together at 30% similarity. Likewise, the core microbiomes from southern flounder and hogchokers do not group at >20% similarity. Core groups of the herbivorous and omnivorous species of adult pinfish, mummichogs, and hogchokers were >40% similar.

Table 3. Alpha diversity metrics (means and SD) for the gut microbiome of each fish species, indicating richness (Chao1 and Observed Species), phylogenetic diversity (PD Whole Tree) and species diversity (Shannon). Indices were calculated at 2000 sequences per sample

| Species | Chao1 | Observed Species | PD Whole Tree | Shannon |
|---|---|---|---|---|
| Cultured mummichog | 124 (10.1) | 104 (16.8) | 11.1 (1.02) | 4.03 (0.82) |
| Wild mummichog | 226 (59.7) | 165 (42.2) | 16.1 (4.01) | 4.53 (1.06) |
| Juvenile pinfish | 159 (37.4) | 95.5 (27.1) | 10.4 (2.04) | 2.83 (0.62) |
| Adult pinfish | 114 (93.6) | 85.7 (73.9) | 8.84 (4.95) | 2.94 (1.66) |
| Silver perch | 67.2 (29.7) | 43.9 (26.7) | 5.19 (3.34) | 2.29 (0.42) |
| Black sea bass | 120 (80.7) | 90.3 (80.3) | 9.78 (7.39) | 3.10 (2.76) |
| Hogchoker | 154 (42.0) | 111 (24.8) | 12.6 (1.97) | 3.20 (0.69) |
| Southern flounder | 110 (52.9) | 85.9 (47.5) | 9.00 (4.62) | 3.54 (1.25) |
| Spanish mackerel | 141 | 132 | 11.9 | 5.57 |
| King mackerel | 143 (42.4) | 82.9 (28.4) | 9.08 (2.57) | 1.79 (0.05) |
| Red drum | 243 (257) | 134 (121) | 12.3 (9.8) | 3.68 (1.71) |
| Crevalle jack | 160 (47.7) | 127 (55.4) | 11.8 (4.40) | 4.24 (2.59) |
| Mahi-mahi | 22.7 (6.34) | 14.0 (3.42) | 2.75 (0.23) | 1.21 (0.89) |
| Great barracuda | 28.2 (0.99) | 14.5 (5.44) | 2.9 (0.72) | 0.69 (0.87) |
| Sharpnose shark | 87.3 (78.8) | 61.6 (61.4) | 7.46 (5.43) | 1.71 (1.12) |
| Spinner shark | 107 (102) | 66.5 (55.9) | 7.32 (5.63) | 2.31 (0.11) |
| Sandbar shark | 28.6 (29.9) | 14.9 (11.9) | 2.47 (1.61) | 0.25 (0.13) |

### 16S rRNA pyrosequencing and Sanger sequencing

Distributions of OTUs similar to those obtained by pyrosequencing were also observed when PCR amplicons from the gut microflora of mummichog, silver perch, pinfish, black sea bass, striped burrfish, Atlantic sharpnose shark, spinner shark, Japanese medaka, red drum, speckled sea trout *Cynoscion nebulosus*, southern flounder, and pipefish *Syngnathus scovelli* were cloned and sequenced. These clone libraries were dominated by OTUs associated with *Proteobacteria* and *Firmicutes* (Fig. S3 in the Supplement). Within the *Proteobacteria*, most OTUs were assigned to γ-*Proteobacteria* within the *Vibrio* and *Photobacterium* genera. Within the *Firmicutes*,

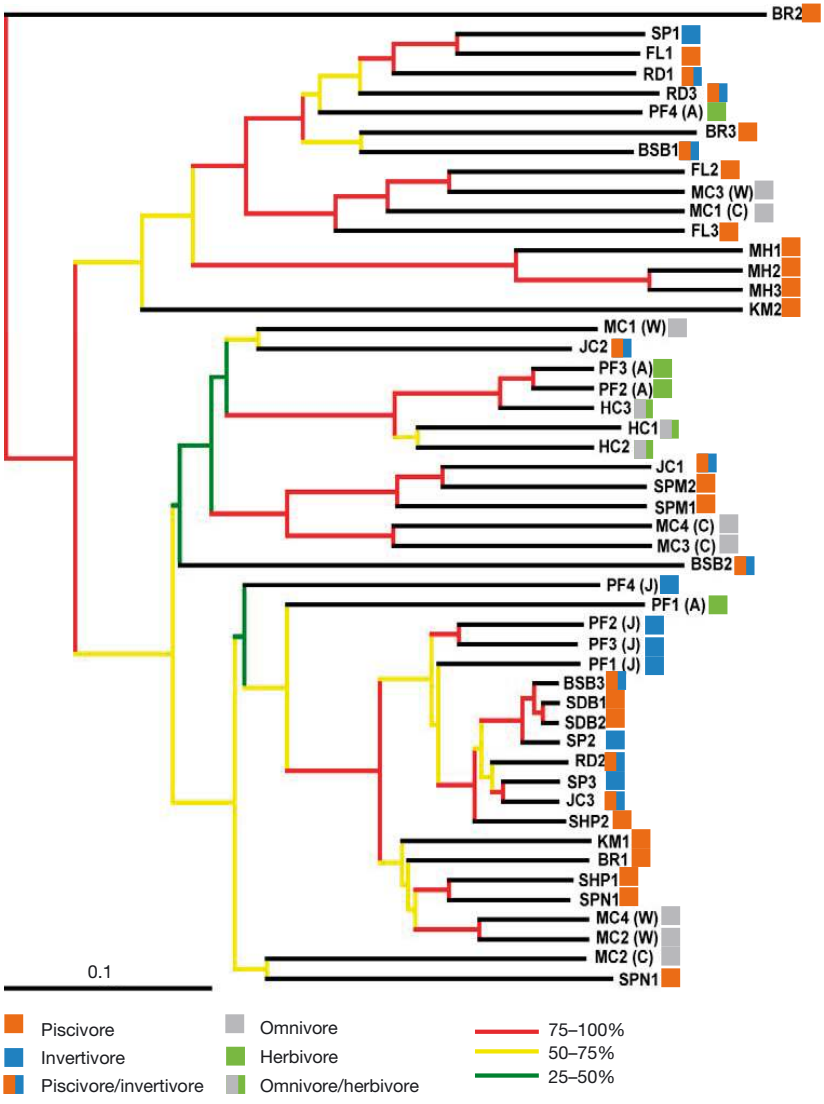


Fig. 2. Differences in the composition of gut microbial assemblages among fish species. Cluster analysis with jackknife support was based on weighted UniFrac distances and UPGMA clustering. Red-colored nodes had 75–100% support, yellow-colored nodes had 50–75% support, and green-colored nodes had 25–50% support. Weighted UniFrac distances were calculated from operational taxonomic units defined at 97% similarity. Species abbreviations are the same as in Table 1; C: cultured, W: wild, J: juvenile, A: adult. Colored squares indicate feeding strategy as defined in Table 1

*Clostridium* sp. OTUs were found in clone libraries of many sampled fish. There were additional contributions from *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlorofexi*, *Fusobacteria*, *Spirochaetes*, *Tenericutes*, and *Verrucomicrobia* in some clone libraries.

Additionally, preliminary clone libraries for pinfish and silver perch were created from fish collected in the summer of 2006 (Ransom 2008), with subsequent collections for clone libraries in the summer 2008 (silver perch) and spring 2009 (pinfish). Pinfish clone libraries included OTUs from 4 (2006) and 11 fish (2009). The 2006 and 2009 pinfish clone libraries were diverse, with contributions from *Proteobacteria*, *Firmicutes*, and *Tenericutes*. Both clone libraries included OTUs from β-*Proteobacteria* and γ-*Proteobacteria*; however, the 2006 library had a greater percentage of β-*Proteobacteria* (mainly *Ralstonia* sp.) and *Firmicutes* (mainly *Clostridia* sp.) than the 2009 library. OTUs similar (>97%) to *Vibrio* spp. and *Enterovibrio* sp. were prevalent within the 2009 library. The silver perch clone library from 2006 was constructed from one sample and was dominated by γ-*Proteobacteria* most closely affiliated with *Escherichia coli.* The 2008 clone library included 3 fish and was dominated by *Clostridium* sp. OTUs, with additional contributions from γ-*Proteobacteria* and *Photobacterium* spp.

### Comparison of cultured and wild mummichogs

Sequences retrieved from cultured and wild mummichogs were distributed among 11 and 12 phyla, respectively (Fig. 4a). *Proteobacteria* OTUs dominated the samples (48 ± 11% and 72 ± 21%, mean ± SD of all sequences retrieved for cultured versus wild fish, respectively). OTUs from the phyla *Actinobacteria*, *Fusobacteria*, *Firmicutes*, and *Bacteroidetes* were also present. Sequences from *Planctomycetes* were found in greater relative abundance in cultured fish (2.5%), while those from *Tenericutes* (1.0%) were more abundant in wild mummichogs.

Within the phylum *Proteobacteria*, 67% (±27%) of all sequences from cultured fish and 74% (±23%) of the all sequences from wild fish were assigned to the γ-*Proteobacteria*. OTUs classified as δ-*Proteobacteria* were only retrieved from wild fish (Fig. 4b). Sequences assigned to *Vibrionaceae* accounted for 19% (±24%) of the gut microflora of cultured mummichogs and 39% (±25%) of the gut microflora of wild mummichogs. Of the *Vibrionaceae*, 99% (±24%) and 84% (±24%) of the

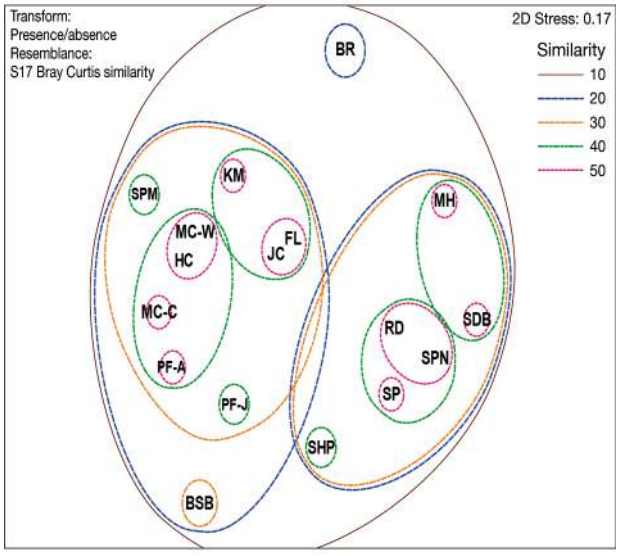


Fig. 3. Non-metric multidimensional scaling comparison of core groups (shared operational taxonomic units [OTUs] among each species) from the gut microbiomes of 12 bony fish and 3 shark species. Data were transformed as presence/absence with Bray-Curtis similarity resemblance, then hierarchical clustering of the Bray-Curtis similarities was performed using the CLUSTER method of the PRIMER software. This information has been superimposed onto the 2-dimensional MDS plot at similarity levels of 20 to 50%. Thus, the bony fish and shark species grouped within similarity circles shared a core group of OTUs that was similar at the levels indicated by the lines enclosing that group of species

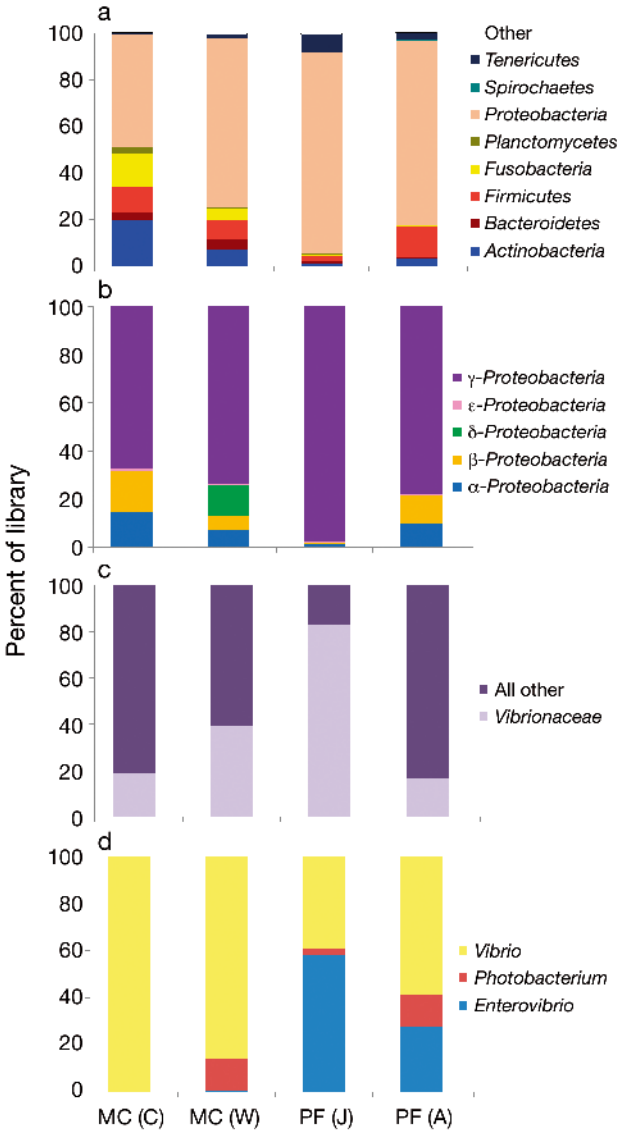


Fig. 4. Composition of the gut microbiome in cultured and wild mummichog (MC(C) and MC(W), respectively), and juvenile and adult pinfish (PF(J) and PF(A), respectively) at 4 levels of taxonomic resolution: (a) phylum level, (b) within *Proteobacteria*, (c) within γ-*Proteobacteria*, and (d) within *Vibrionaceae*. In all cases, composition is expressed as percentages of sequences retrieved. The 'Other' group in (a) includes all phyla contributing <1% of total sequences

sequences retrieved from the gut microflora of cultured and wild fish, respectively, were binned to the genus *Vibrio* (Fig. 4d).

The core gut microbiomes of cultured and wild mummichogs contained 27 and 41 OTUs, respectively, including 12 shared OTUs that were distributed among the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. There is >75% jackknifed support for the cluster that contains wild mummichog specimens 1, 2, and 4 (Fig. S4 in the Supplement), while cultured mummichog specimens 3 and 4 cluster together with >75% jackknifed support. The microflora of cultured and wild mummichogs was significantly different (ANOSIM, R = 0.67, p = 0.02).

### Comparison of juvenile and adult pinfish

Sequences retrieved from juvenile and adult pinfish were assigned to 11 and 10 phyla, respectively (Fig. 4a). *Proteobacteria* OTUs dominated both groups, accounting for 87 ± 15% (juvenile) and 79 ± 32% (adult) of the sequences retrieved. OTUs representing *Actinobacteria*, *Firmicutes*, and *Tenericutes* were present in both groups but at lower relative abundances. *Spirochaetes* OTUs contributed 1% of the sequences found in adult fish, but were not detected in juvenile fish.

The *Proteobacteria* OTUs retrieved from juvenile *Lagodon rhomboides* gut microbiomes were predominantly γ-*Proteobacteria*, whereas adults had addi-

tional contributions from β-*Proteobacteria* (Fig. 4b). On average, 83% (±14%) of the *Proteobacteria* sequences retrieved from juvenile pinfish were assigned to the family *Vibrionaceae*, with those sequences divided amongst the genera *Enterovibrio* (35 ± 20%) and *Vibrio* (23 ± 35%). *Vibrionaceae* OTUs only accounted for 17% (±24%) of the sequences retrieved from adult pinfish (Fig. 4c), and these could be further sorted into *Enterovibrio* (4.0 ± 6.9%) and *Vibrio* (8.4 ± 13%).

Juvenile and adult pinfish shared a core gut microflora consisting of 9 OTUs. The core group of juvenile pinfish contained 43 OTUs, but the adult core group only contained 14 OTUs. The main difference between the core groups of juvenile and adult pinfish was the presence of *Enterovibrio* sp., *Vibrio* sp., and *Rhodobacterales* OTUs in the juvenile core group. The core group of adult pinfish also included *Halomonas* sp. and *Sphingomonas* sp., neither of which was found in the core group of juvenile *L. rhomboides*.

The gut microflora of adult and juvenile pinfish clustered together with >75% jackknifed support (Fig. S5 in the Supplement). However, the microflora of some of the juvenile pinfish form an additional cluster (with >75% support) that do not include adult pinfish. Juvenile pinfish specimen 4 and adult pinfish specimen 4 clustered separately from the other pinfish samples. These 2 fish had an 'intermediate' body length compared to the other pinfish samples, suggesting that they may have been in transition from juvenile to adult digestive physiologies, with concomitant changes in microflora composition. Juvenile, intermediate, and adult pinfish gut microflora assemblages were significantly different from each other (ANOSIM, R = 0.77, p = 0.004).

## DISCUSSION

The continued development and advancement of techniques in marine microbial ecology from plating to clone libraries to today's high-throughput sequencing technology have facilitated studies dedicated to characterizing gut microflora and understanding its influence in microbe–host interactions (summarized in Table S1). As with studies of diversity in bacterioplankton communities (Sogin et al. 2006), results from using high-throughput sequencing in our analysis suggest that microbiomes of both bony fish and shark guts harbor more diversity than suggested by earlier studies using culture-dependent methods or analysing cloned 16S rRNA amplicons (Newman et al. 1972, Grimes et al. 1985, MacFarlane et al. 1986, Spangaard et al. 2000, Verner-Jeffreys et al. 2003, Ransom 2008). We recovered OTUs distributed among 7 to 15 different phyla per species. Several phyla (*Acidobacteria*, *Calditrix*, *Chlorobi*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, *Thermi*, and *Verrucomicrobia*) were minor, rare components (<1% of the sequences retrieved) of the gut microbiomes of several fish species. For all fish species, richness ranged from 2 to 6 dominant (>1%) phyla. The dominance of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Planctomycetes*, *Proteobacteria*, and *Tenericutes* was consistent with that reported in a meta-analysis of published studies of fish gut microbiomes based on analyses of clone libraries (Sullam et al. 2012). Sequences from all of these phyla except *Tenericutes* were also detected at varying contributions in the gut microbiome of the zebrafish *Danio rerio* (Roeselers et al. 2011) and the common carp *Cyprinus carpio* (van Kessel et al. 2011) in studies using 454-pyrosequencing.

*Firmicutes* and *Bacteroidetes* OTUs dominate the terrestrial mammalian gut microbiome at 65.7 and 16.3% of all sequences retrieved for all samples, while sequences assigned to *Proteobacteria* were much less common (8.8% of total) (Ley et al. 2008a). In contrast, *Proteobacteria* (62.5% of all sequences retrieved from all samples) dominated the fish gut microbiome in our study, with smaller contributions from sequences assigned to the phyla *Firmicutes* (14.2%) and *Bacteroidetes* (1.03%). This difference between the gut microbiomes of fish and terrestrial mammals (Ley et al. 2008a,b) could be attributed to a legacy effect (Rawls et al. 2006), stemming from the very different environments inhabited by fish versus terrestrial mammals, or to parental inheritance. Rawls et al. (2006) further theorized that both legacy effects and differences in the gut habitat (including differences in gut anatomy, physiology, immunology and nutrient composition) contribute to the divergent gut microbiomes of fish and mammals.

Legacy and difference in gut habitat may also explain inter- and intraspecific differences in gut microflora composition. Our results suggest that within-species variability in the composition of the gut microbiome is significant in some species (i.e. barracuda). This variability has been documented in studies of mammalian gut microflora (Ley et al. 2008b, De Filippo et al. 2010, Yatsunenko et al. 2012) and suggests that the composition of the gut microflora community responds to external factors such as habitat and diet, including bacteria introduced into the gut with the diet. In a gut microflora

transplant experiment between mice and zebrafish, Rawls et al. (2006) concluded that both the fish and mouse gut provide suitable habitats and niches for the transplanted microbial assemblage, which then evolves in response to host-specific physiology and metabolic needs.

As in previous studies of fish gut microflora, *Proteobacteria* OTUs dominated (>50% of the sequences retrieved) the gut microbiomes of 67% of the fish species we sampled, suggesting that they are a core component of most fish species' gut microflora. Within a given species, individual fish contained the same phyla (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*), but at varying contributions to the total gut microflora community. As suggested in previous studies of gut microflora (Eckburg et al. 2005, Tap et al. 2009, Roeselers et al. 2011), these phyla likely represent a 'core' bacterial community. If the core gut microflora is defined by the OTUs (>97% similarity) found in all samples of a given species, we did not find a core microbial assemblage that encompassed all of the fish species we sampled. This is not surprising, considering that the mammalian gut microbiome (Ley et al. 2008a,b) did not share a single OTU (≥96% similarity) among all mammalian species sampled (humans and 59 terrestrial mammals). Our results suggest that the core gut microflora of each fish species assembles in response to the fish's specific physiological demands and dietary constraints.

Although no single OTU was shared among all fish species, many of the OTUs present in one species' core group were also found in the core groups of other species. Several OTUs were consistently found in the guts of multiple species, suggesting that these OTUs play an important functional role in microflora assemblages.

*Firmicutes*, *Fusobacteria*, *Spirochaetes*, and *Tenericutes*, but not *Proteobacteria*, were the dominant OTUs found in the guts of barracuda, mahi-mahi, king mackerel, Spanish mackerel, and southern flounder. The gut microflora community from mahi-mahi was dominated (83% of the sequences retrieved) by *Spirochaetes* OTUs, most of which were assigned to the genus *Brachyspira* (91%). The different mahi-mahi samples were not collected from the same location or at the same time; thus, the dominance of *Spirochaetes* OTUs in all 3 samples indicates that *Spirochaetes* and *Brachyspira* sp. are core members of the mahi-mahi gut microflora. The genus *Brachyspira* is known as an 'intestinal spirochaete' and has been documented as a gut pathogen in pigs (Hampson & Ahmed 2009). These bacteria have also been reported in the intestinal tract of various mammals (including humans) and birds, and the genus includes species that are commensals and pathogens (Bellgard et al. 2009). Our sequence data do not allow us to determine which *Brachyspira* species were present; however, the mahi-mahi we sampled did not display any signs of impaired health when captured.

Previous applications of culture-independent techniques for examining gut microflora have revealed that *Mycoplasma* sp. are abundant in the gut microflora of a variety of hosts (Giebel et al. 1990, Holben et al. 2002, Hongoh et al. 2003, Gulmann 2004, Tanaka et al. 2004, Bano et al. 2007, Ward et al. 2009, Meziti et al. 2010). *Tenericutes* OTUs were recovered from several fish in this study; however, they were only members of the core group of 2 species, king mackerel and mahi-mahi. The contribution of *Mycoplasma* sp. OTUs to the pinfish gut microbiome was variable, ranging from 0 to 30% of sequences retrieved, suggesting that their presence within this species' gut is influenced by environmental factors, likely diet in this omnivorous species.

The core gut microflora of all 3 shark species sampled shared 3 OTUs, with *Photobacterium* OTUs dominating. This is consistent with Grimes et al. (1985), who used culture-dependent methods and reported that *P. damselae* is a normal member of the gut microflora of sharks. Our work expands on the Grimes et al. (1985) study with deeper coverage and the application of a culture-independent technique. Our data indicate that *Actinobacteria*, *Firmicutes* (*Clostridium* sp), *Fusobacteria* (*Cetobacterium* sp.), and other *Proteobacteria* (*Campylobacter* sp. and *Vibrio* sp.) are also important members of the shark gut microbiome.

The results of our comparison of the gut microflora of cultured versus wild mummichogs are consistent with those of Roeselers et al. (2011), who found that, although the composition of gut microflora communities of cultured versus wild *Danio rerio* differed, they still shared a core group of microflora. Our results also agreed with those of MacFarlane et al. (1986) in that the gut microbiome of wild mummichogs was richer, more diverse, and had a larger core group than their cultured (i.e. farmed) counterpart. Cultured mummichogs are fed a regimented diet and reside in a controlled environment, whereas wild mummichogs are opportunistic omnivores and inhabit an estuarine ecosystem with variable salinity, temperature and potential food items. We suggest that wild mummichogs have a diverse gut microflora that allows them to satisfy the physiological require-

ments of a changing intertidal environment and the metabolic needs of a varied omnivorous diet.

Our results also indicate that differences in the pinfish core gut microbiome correlate with age-size classes (juvenile to adult) and with an ontogenetic shift from a primarily carnivorous diet to herbivory. The gut microflora of intermediate-sized pinfish was statistically significantly different from that of both juvenile and adult fish and contained a transitional microflora, suggesting that their gut microflora has adapted for an 'intermediate' diet. These findings are consistent with Luczkovich & Stellwag (1993), who found qualitative shifts in pinfish gut microflora correlating with the transition from juvenile to adult.

Ley et al. (2008a) concluded that gut microflora of herbivorous mammals have the greatest richness and phylogenetic diversity, and that both richness and phylogenetic diversity decreased among omnivores and decreased further among carnivores. We found lowest richness and phylogenetic diversity (Table 3) in gut microbiomes from fish defined as top piscivores (carnivores; e.g. mahi-mahi, barracuda, and all shark species) (Froese & Pauly 2011). Although southern flounder, king mackerel, and Spanish mackerel are also reported to be piscivores (Froese & Pauly 2011), their gut microbiomes were richer and more diverse than those of the top piscivores. We found no statistically significant difference between the richness of fish defined as piscivores (Kruskal-Wallis ANOVA). However, there was a statistically significant difference in the calculated richness between species classified as invertivores/piscivores and piscivores (Chao1, $p = 0.05$) and those classified as omnivores and piscivores (Chao 1, $p = 0.02$; Observed Species, $p = 0.006$), suggesting that gut microflora richness may be linked to a more varied diet. Feeding studies we conducted (Givens 2012) showed that diet influenced the composition of fish gut microflora, especially the transient (or non-core) microflora assemblage. The influence of diet on gut microflora composition was especially evident in herbivorous and omnivorous fish species that had large contributions from chloroplast sequences to microbiomes that we recovered from their guts (Givens 2012, present study). The presence of large numbers of chloroplast sequences suggests that DNA in cells associated with food can contribute significantly to the OTUs recovered from a sample. Whether these cells are active and contribute to digestion or other gut functions is not known; however, the chloroplast example suggests that they may simply represent undigested food.

In conclusion, we found that increased richness and diversity of the gut microbiome correlated with a more varied diet. The gut microbiomes of wild mummichogs, the most omnivorous of the fish we sampled, had the greatest richness (Chao1 and Observed Species), highest phylogenetic diversity (PD Whole Tree), and second highest Shannon diversity. However, the relationship between the richness of gut microbiomes and feeding strategy does not appear to be as clearly delineated in fish as in mammals (Ley et al. 2008a). An important distinction between our study and Ley et al. (2008a) is that most of the mammalian gut microbiome samples came from captive animals that were fed a relatively unvarying daily diet. With the exception of samples from cultured mummichogs, all our samples came from wild-caught fish, which are likely consuming a more varied diet than captive animals. Fish with diverse diets may support a richer gut microflora assemblage as a result of the wider range of potential substrates available to the core microbial community and the greater diversity of microbiota inoculated into the gut from different food sources. The host may contribute to community assembly by selecting for microbial populations that include specialized bacteria to aid in the digestion of and absorption of nutrients from a variety of food sources (i.e. protein versus chitin or structural polysaccharides).

The intraspecies variability in microflora community composition suggests that the gut microbiomes of individual fish may respond to changing environmental factors (i.e. water temperature, salinity) and especially to diet. Diet-associated bacteria (i.e. bacteria living in or on food consumed by an individual) may contribute to the gut microflora, either as inocula for the resident core population or as transients that are flushed out once diet changes. The fish gut microbiome contributes to digestion and can affect nutrition, growth, reproduction, overall population dynamics, and vulnerability of the host fish to disease (MacFarlane et al. 1986). Understanding how these functions change in response to differences in composition is an important next step to predicting how the host-gut microflora consortium will function in a changing environment and to understanding and managing fish health.

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