INTESTINAL MICROBIAL COMMUNITY COMPOSITION OF SIX ACTINOPTERYGII FISH SPECIES IN THE SOUTHEASTERN UNITED STATES

by

BRIANA LEE RANSOM

(Under the Direction of James T. Hollibaugh)

ABSTRACT

The intestines of fish harbor a microbial community that aids digestion and prevents colonization by pathogens. Traditional methods of studying these communities have been cultivation dependent, yet many microorganisms are difficult to grow in the laboratory. In this study, gut microflora of six different Actinopterygii fish species from the southeastern United States were examined by PCR/DGGE analysis and cloning with primers specific for the 16S rRNA genes of Bacteria. Most fish species seem to have gut microflora dominated by either Firmicutes or proteobacteria. Sequences 92-93% similar to species of *Mycoplasma* were found in pinfish (*Lagodon rhomboides*) and red drum (*Sciaenops ocellatus*), while flounder (*Paralichthys lethostigma*) contained a majority of sequences most similar to *Clostridium* spp. The gut microflora of pipefish (*Syngnathus scovelli*) and silver perch (*Bairdiella chrysoura*) were dominated by members of the division proteobacteria, and speckled trout (*Cynoscion nebulosus*) were not dominated by either Firmicutes or proteobacteria.

INDEX WORDS: Gut microflora, PCR, DGGE, Cloning, Proteobacteria, Firmicute, *Mycoplasma*, *Clostridium*

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BRIANA LEE RANSOM

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BRIANA LEE RANSOM

Major Professor: James T. Hollibaugh

Committee: Duncan Krause
Mary Ann Moran

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

For a love that endures even when the body cannot, I dedicate this thesis to my mother, Phyllis Lee Ransom. You are truly always in my heart.

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CHAPTER 1

INTRODUCTION

The digestive tracts of fish, like those of higher vertebrates, harbor a distinct microbial community. Intestinal microflora play an important role in the health of the fish by aiding in digestion (Cahill 1990; Stellwag, Smith et al. 1995; Mountfort, Campbell et al. 2002; Moran, Turner et al. 2005) and by promoting disease resistance (Hansen and Olafsen 1999; Spanggaard, Huber et al. 2000). A thorough understanding of intestinal endosymbionts is of great importance to the aquaculture industry. Studies of fish raised in water that contained antibiotics have demonstrated that once the original microbial community is eliminated from the gut, a different community will take its place, suggesting that the original community may have conferred resistance to microbial pathogens (Cahill 1990). There is also evidence from experiments with gnotobiotic zebrafish that the host plays a role in determining the composition of the gut microflora (Rawls, Mahowald et al. 2006).

Traditionally all studies of fish intestinal microflora were, by necessity, conducted using culture-dependent identification and quantification methods. Not surprisingly, these studies were limited in scope to those microorganisms that are easily cultured in a laboratory setting. It is notoriously difficult to culture many microorganisms from environmental samples and therefore culture-based methods may account for only 1% of the active bacteria in the sample (Amann, Ludwig et al. 1995; Romero and Navarrete 2006) and are certainly biased by the conditions under which the cultures are grown (Cahill 1990). In studies comparing culture-based and molecular bacterial quantification methods in the intestine of the rainbow trout (*Oncorhynchus*

mykiss), the numbers obtained from aerobic plate counts were 50-90% of the numbers obtained from direct microscope counts (Spanggaard, Huber et al. 2000; Huber, Spanggaard et al. 2004). This study suggests a more optimistic view of the culturability of gut microflora, though it remains unlikely that traditional culture-based methods accurately represent the full microbial diversity in fish intestines. Organisms commonly identified using the traditional culture-based methods include *Enterobacter, Aeromonas, Pseudomonas, Acinetobacter, Flavobacterium,* and *Vibro* species (Cahill 1990; Ringø, Strøm et al. 1995).

More recent studies have used molecular methods to identify gut microflora and have been able to detect microorganisms previously undocumented in fish guts. Holben et al. (2002) used molecular methods to identify a novel mycoplasma phylotype in the intestines of Atlantic salmon (*Salmo salar*) and Bano et al. (2007) identified an abundance of mycoplasma ribotypes in long-jawed mudsuckers (*Gillichthys mirabilis*). Both of these studies reported that a majority (up to 96%) of the bacteria in the gut of each species belonged to the genus *Mycoplasma*. The fact that mycoplasmas had not been widely reported as being significant components of the gut microflora using the previous culture-based methods suggests that other important components of gut microbiota may as yet be undiscovered.

The current study aims to describe the intestinal microflora of a variety of fish species common to the southeastern United States using molecular identification techniques such as PCR/DGGE analysis and cloning/sequencing with the objective of determining whether or not mycoplasmas are as widespread a component of intestinal microflora as suggested by analysis of salmon (Holben, Williams et al. 2002) and mudsuckers (Bano, Smith et al. 2007). The species in this survey include nine pinfish (*Lagodon rhomboides*), one silver perch (*Bairdiella chrysoura*), six pipefish (*Syngnathus scovelli*), two southern flounder (*Paralichthys lethostigma*), two red

drum (*Sciaenops ocellatus*), and one speckled trout (*Cynoscion nebulosus*). These fish represent three different orders of Actinopterygiian (ray-finned) fishes: Perciformes, Syngnathiformes, and Pleuronectiformes. Figure 1 shows the evolutionary relationships between the major orders of teleost fishes as described in *Ichthyology Handbook* (Kapoor and Khanna 2004). Pinfish, silver perch, red drum, and speckled trout belong to Percomorpha (also called Perciformes); not shown in this figure are the orders Syngnathiformes (pipefish) and Pleuronectiformes (flounder), both of which belong within superorder Acanthopterygii.

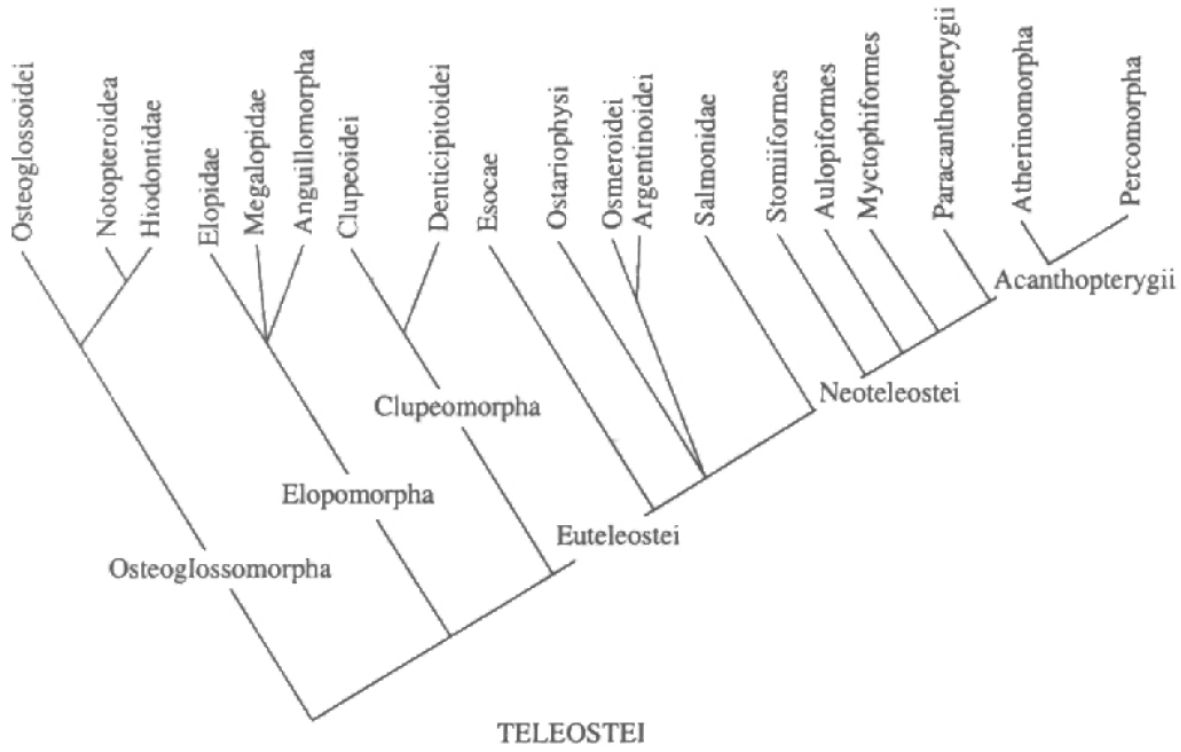


Figure 1. Tree depicting evolutionary relationships between major orders and superorders of teleost fishes (Kapoor and Khanna 2004).

CHAPTER 2

EXPERIMENTAL PROCEDURE

SAMPLE COLLECTION

All fish used in this study were collected in the summer of 2006 from the Florida and Georgia coasts. The pinfish and silver perch were collected in a series of two surface trawls at the Florida State University Coastal and Marine Laboratory (FSUCML; Trawl 1: 29°54'23.6"N, 84°31'16.8"W, Trawl 2: 29°54'7.1"N, 84°31'36.8"W). This location was approximately 1.5 km offshore and was characterized by a mix of sandy substrate and seagrass beds and a salinity of 35. Before trawling, 300 ml of surface water was filtered through two 0.2 μm pore size Sterivex™ cartridge filters (Millipore, Billerica, MA). DNA extracted from these filters was amplified and run on DGGE alongside the DNA extracted from the fish intestines in order to determine the similarity of the gut microflora and the microbial community of the surrounding water. Fish were held in plastic tubs filled with seawater until return to shore. Water was changed periodically to reduce stress to fish. On shore, the fish were placed in tanks with recirculating seawater until guts could be harvested (~10 hr). They were not fed during this time. Fish were killed by decapitation and the intestines were removed immediately, placed in sterile phosphate buffered saline (PBS), and frozen at –20 °C. If digesta was present in the intestines, it was squeezed into a separate tube and frozen in sterile PBS so that the microbial community present in the digesta could be compared to the flora of the gut wall. However, this method does

not result in a clean separation and members of the digesta community may remain on the gut wall and vice versa.

Pipefish were obtained from Gulf Specimens Marine Lab (Panacea, FL). Freshly caught fish were placed directly on ice and frozen before being shipped on dry ice to Athens, GA. Fish were dissected, the entire gut was removed and digesta was collected into a sterile tube containing PBS, then the intestine was placed in sterile PBS. The southern flounder, red drum, and speckled trout were caught on a hook and line around the southern end of Sapelo Island, Georgia (31°22'30"N, 81°16'30"W), and the guts were harvested immediately and placed in sterile PBS. Digestive tracts from the flounder, red drum, and speckled trout were separated into three sections before freezing: an anterior section from near the throat, a foregut region posterior to the stomach, and a hindgut region ending at the anus. All samples were stored at –20 °C until processed.

DNA EXTRACTION

Tubes containing intestines were allowed to come to room temperature. Intestines were opened with a flame-sterilized scalpel if they had not been split before freezing. Tubes were sonicated on ice for 15 minutes to detach bacteria from the walls of the intestine. After sonication, supernatant was poured into a clean tube. Fresh PBS was added to the tube containing the gut section, shaken vigorously, and sonicated on ice for an additional 15 minutes to remove any remaining bacteria. This supernatant was combined with the supernatant from first sonication, then tubes were centrifuged at 15,000 ×g to pellet the bacteria. The supernatant was decanted into the original tube containing the gut section and stored at –20 °C. Sterivex cartridges were broken open, the filters were removed with a flame-sterilized scalpel and forceps

and placed into a test tube. Bacteria in the pellet and sterivex filters were then extracted using a MoBio PowerSoil DNA Extraction Kit (Solana Beach, CA), following all manufacturer's instructions.

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE analysis used a nested amplification protocol to reduce the possibility of nonspecific amplification of eukaryotic (fish) DNA by DGGE primers. First, Bacteria DNA was amplified using Ready-To-Go™ PCR beads (Amersham Biosciences; Piscataway, NJ) with universal Bacteria primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). One µl of undiluted DNA template was added to each tube. After an initial denaturation at 95 °C for 5 min., there were 20 cycles of: denaturation at 95 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min.; followed by a final extension at 72 °C for 45 min. The products of this PCR reaction were then amplified using the Bacteria DGGE primer 356F (5'-CCTACGGGAGGCAGCAG-3') with an added GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCC-3'; Myers, Fischer et al. 1985) and the universal primer 517R (5'-ATTACCGCGGCTGCTGG-3') labeled with fluorescein. All primers were manufactured by Operon Biotechnologies (Huntsville, AL).

In addition to the 356F/517R DGGE primer set, a mycoplasma-specific primer set was used to obtain a more precise assessment of the mycoplasma population present in the gut samples. Primer 356F with the GC clamp was used as described above, but primer 517R was replaced with the mycoplasma primer 543R (5'-ACCTATGTATTACCGCG-3'; McAuliffe, Ellis et al. 2005), labeled with fluorescein. PCR conditions were as described by McAuliffe et al. (2005). Primer 543R was designed after aligning 102 *Mycoplasma* sequences and yielded

positive amplification with all 72 samples used for testing (McAuliffe, Ellis et al. 2005). The positive control for this reaction was a previously analyzed mudsucker gut sample known to contain mycoplasmas (Bano, Smith et al. 2007), and nuclease-free water was used as a negative control.

The mixed template amplicons were resolved by DGGE using a CBS Scientific (Del Mar, CA) system as described previously (Ferrari and Hollibaugh 1999), with a denaturant gradient of 40% to 80%. Gels were read on a Hitachi FMBio II® fluorescence imaging system and bands of interest were excised with a flame-sterilized scalpel. DNA was extracted from the gel slices in 50 µl of nuclease-free water incubated at 50 °C for one hour. If bands in the original gel were not clear, the DNA they contained was reamplified with 356F/517R under the same conditions, resolved on a second DGGE, and excised again. DNA from clearly defined bands was amplified with 356F/517R (without the GC clamp or fluorescein), then purified using a QIAQuick® PCR purification kit (QIAGEN; Valencia, CA). A BigDye® terminator cycle sequencing kit (Applied Biosystems; Foster City, CA) was used for sequencing by the University of Georgia Integrated Biotech Laboratory on an ABI 3100 Genetic Analyzer® (Applied Biosystems).

CLONING

Using the DGGE community fingerprint as a guide, one sample containing representative bands for each fish species was chosen for the creation of a clone library. DNA from the six fish samples was amplified using Bacteria primers 27F and 1492R and the DNA from the FSUCML water sample was amplified with mycoplasma-specific primers in order to determine if mycoplasmas were present in the water column. PCR product from all samples was run on an agarose gel and bands of the appropriate length (~1400 bp for the universal primers and ~200 bp

for the mycoplasma-specific primers) were excised with a flame-sterilized scalpel. Gel fragments were purified using a QIAQuick® gel extraction kit (QIAGEN; Valencia, CA), following the manufacturer's instructions and performing the final elution in 30 µl of nuclease-free water.

The cloning reaction was performed using a TOPO TA® cloning kit for sequencing (Invitrogen; Carlsbad, CA) using the pCR®4-TOPO vector and One Shot TOP10® competent cells. The cloning reaction consisted of 2 µl DNA template, 0.5 µl salt solution, and 0.5 µl of vector and was incubated at room temperature for 30 minutes. The transformed cells were plated onto LB plates containing 50 µg/ml ampicillin and spread with 40 ul of 40 mg/ml X-gal, then incubated at 37 °C overnight. After incubation, the plates were placed at 4 °C for 4-6 hours to stop the growth of the colonies and allow color to develop. Colonies containing inserts were grown in a 96-well block containing LB with glycerol added to facilitate long-term storage at –80 °C. Between 24 and 32 colonies were picked for each fish species. Clone libraries were compared using webLIBSHUFF (Henriksen 2004), an online interface for the LIBSHUFF program developed by Singleton et al. (2001) to test the statistical differences between them.

After the creation of the initial clone libraries, two fish species were chosen for further investigation based on differences detected in the composition of their gut microflora. Because of the dominance of mycoplasmas in the first set of samples, three additional pinfish samples were selected for the creation of replicate clone libraries; two of these samples (libraries B and C) consisted of digesta only while the third (library D) contained gut tissue as well as digesta. In order to compare these libraries to samples from fish dominated by proteobacteria gut microbiota, three pipefish samples (all digesta only, libraries labeled F, G, H) were also chosen. Cloning was performed as described above and 96 clones from each of the six samples were

picked for sequencing. The webLIBSHUFF program (Henriksen 2004) was used to compare clone libraries to determine if they were significantly different.

DNA SEQUENCING

Clones were sequenced at a commercial facility, SeqWrite (Houston, TX), with the standard primers for the cloning vectors, M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'). Because of the length of the insert (~1450 bp), samples were sequenced in both directions in order to assemble nearly full-length sequences. Sequences were edited and aligned with the Genetics Computer Group Wisconsin Package, version 11. Neighbor-joining trees were created with the PHYLIP package using Jukes-Cantor distances. Bootstrap values were calculated from 100 iterations.

Since the replicate libraries of pinfish and pipefish amplicons were created for the purpose of comparison between samples of the same species, these clones were only sequenced in the forward direction and no full-length sequences were assembled. The same universal Bacteria primer (27F) that was used for cloning was used for sequencing. Phylogenetic trees were created as described above with the GCG Wisconsin Package and PHYLIP.

CHAPTER 3

RESULTS

SILVER PERCH (*BAIRDIELLA CHRYSOURA*)

The gut of the silver perch was empty when it was harvested, so no comparison could be made between digesta versus gut wall microbial communities. Figure 2 shows the composition of the silver perch and pinfish gut microflora, and a water sample taken at the same time the fish were collected. One dominant band contained a DNA sequence that was most similar to an unknown bacterium belonging to the family Enterobacteriaceae (94% similarity) and another dominant band co-migrated with a band in the pinfish samples that contained DNA most closely related to a *Mycoplasma lipofaciens* isolate (84% similarity). Since there was only one silver perch sample, 16S rDNA amplicons from it were used to generate a clone library.

The clone library for the silver perch consisted of 32 sequences, 16 of which (50%) were most similar (>99% similarity) to *Escherichia coli*. Five of the sequences (16%) were most similar (87% similarity) to the chloroplast of an Apicomplexan parasite *Eimeria* sp. These five sequences were significantly different from all of the others used to build a comprehensive phylogenetic tree for all fish species, and therefore were omitted. Of the remaining sequences obtained from the clone library, three (9%) were 99% similar to *Stenotrophomonas maltophila*, and two each (6%) were most similar to *Vibrio* sp. (99% similarity) or *Merismopedia glauca* (93% similarity). One sequence each was most similar to *Staphylococcus gallinarum* (99% similarity), an uncultured Verrucomicrobia (94% similarity), an uncultured Chloroflexi (91%

similarity), and an uncultured γ-proteobacteria (97% similarity). Although there were bands on the DGGE gel that co-migrated with *Mycoplasma* amplicons, none of the sequences obtained from the silver perch clone library were closely related to any mycoplasmas.

RED DRUM (*SCIAENOPS OCELLATUS*)

The anterior and foregut portions of both red drum specimens (labeled A and F, respectively) exhibited very similar banding patterns when resolved on DGGE (Fig. 3). The hindgut portions from both fish appeared to contain a more diverse microbial community. The sample chosen for cloning was one of the hindgut sections that contained all of the dominant bands from the A and F sections, plus two presumed mycoplasma bands and a strong unidentified band. Although the DGGE with primers 356F/517R only showed dominant mycoplasma bands in one red drum hindgut sample, bands that co-migrated with mycoplasma amplicons were obtained from the anterior and foregut samples of the same fish when the mycoplasma-specific primer set was used for PCR/DGGE (Fig. 4). The mycoplasma bands appearing in the anterior and foregut sections were not as prominent as the bands in the hindgut section and bands resulting from amplification by the mycoplasma-specific primers were generally less prominent than those resulting from amplification by 356F/517R. There were no distinct mycoplasma bands present in the anterior or foregut samples of the second red drum, although there appeared to be a very faint band present in the hindgut sample.

A total of 32 red drum clones were picked for sequencing; 16 (56%) of them contained DNA sequences most closely related to *Mycoplasma* sp. Like the presumed mycoplasmas in the pinfish, these sequences were only 92-93% similar to known mycoplasmas and therefore cannot be placed definitively within that genus. Seven sequences (22%) were 95% similar to

Arthromitus sp. found in a diarrhetic rainbow trout intestine, five (16%) were 97-99% similar to *Vibrio* sp., and one (3%) sequence each was most similar to *Bacillus* sp. (97% similarity) and *Escherichia coli* (99% similarity).

SPECKLED TROUT (*CYNOSCION NEBULOSUS*)

As seen in figure 3, only one dominant band was present in all three speckled trout samples (anterior, foregut, and hindgut). The remaining bands in the DGGE were unique to each gut section. The bands were not cleanly which resulted in ambiguous sequences. The hindgut section (H) appeared to have the most diverse microbial community and was therefore selected for cloning.

Twenty-four clones were chosen for sequencing but two of the sequences obtained were not usable. Five sequences (23%) were 99% similar to *Escherichia coli*, three (14%) each were most similar to representatives of the genera *Eubacterium* (95% similarity) and *Clostridium* (97% similarity), and two (9%) each were most similar to representative sequences from *Granulicatella* (98% similarity), *Bacillus* (97% similarity), and *Synechococcus* (96% similarity). One sequence (5%) each was most similar to representatives of *Azospirillum* (94% similarity), *Roseobacter* (93% similarity), an uncultured γ-proteobacterium (98% similarity), a verrucomicrobium (94% similarity), and a cyanobacterium (95% similarity).

FLOUNDER (*PARALICHTHYS LETHOSTIGMA*)

As seen in figure 3, a single dominant band was found in DGGE images of all six flounder samples (anterior, foregut, and hindgut sections of two individuals). Similarly to the red drum, the anterior (A) and foregut (F) samples produced very similar banding patterns while

the hindgut (H) section was the most diverse. In the flounder samples, however, the hindgut section did not contain the same dominant bands found in the other gut sections, so it was not used for the clone library. Instead, one of the foregut sections was chosen.

A total of 32 clones were chosen for sequencing, and 29 of these yielded unambiguous sequences. The majority of these sequences (66%) were most similar (96-99% similarity) to *Clostridium* sp., four (14%) were 99% similar to *Cetobacterium ceti*, two (7%) each were most similar to *Eubacterium tarantellus* (95% similarity) and *Chitinophaga pinensis* (85% similarity), and one (3%) each to an uncultured member of class Flavobacteria (88% similarity) and *Stenotrophomonas maltophila* (99% similarity).

PINFISH (*LAGODON RHOMBOIDES*)

When the pinfish digesta, gut wall, and surrounding water samples were resolved in adjacent lanes on DGGE, additional bands appeared in the digesta that were not present in the samples extracted from the wall of the intestine (Fig. 2). The DNA sequences from these additional bands were most similar (93-94% similar) to members of the genus *Clostridium*. Regardless of whether the DNA was extracted from the digesta or the gut wall, the DGGE gels were dominated by bands that were most closely related to *Clostridium* spp. and *Mycoplasma* spp. One dominant band contained DNA that was most closely related (93% similar) to an uncultured γ-proteobacterium. The microbial community of the surrounding water had a distinctly different banding pattern than the pinfish gut samples. The dominant *Clostridium* species found in the digesta and gut tissue samples were not present in the water column, nor was the dominant γ-proteobacterium.

Pinfish digesta, gut wall, and surrounding water samples were amplified with a mycoplasma-specific primer and run on a DGGE alongside those samples amplified with 365F/517R (Fig. 1). The dominant bands resulting from both primer sets were very similar, with the exception of the single γ-proteobacteria band amplified with 365F/517R that did not amplify when using the mycoplasma-specific primer set. Because the mycoplasma-specific primer set amplified non-mycoplasma DNA, it by itself was not a reliable indicator of mycoplasmas.

Examination of DGGE banding patterns led to the selection of sample PN11W for the creation of the initial clone library (A). The DGGE from this sample contained the dominant mycoplasma bands as well as the dominant γ-proteobacteria, but not *Clostridium*. A total of 24 clones were picked for sequencing; one of them returned an unreadable sequence. Of the remaining 23 sequences, 16 of them (~67%) were most closely related to members of the *Mycoplasma* genus. However, these sequences were only 92-93% similar to published *Mycoplasma* sequences, so it is unclear whether they actually fall within the *Mycoplasma* genus. Of the remaining sequences obtained by cloning, four (~17%) were most closely related to an uncultured γ-proteobacterium (98% similarity) and one sequence each (~4% of the library) was most similar to *Ureaplasma urealyticum* (90% similarity), *Delftia acidovorans* (99% similarity), and *Eubacterium tarantellus* (95% similarity).

Figure 6 depicts representative sequences from the four pinfish clone libraries and their relative abundance in each library. None of the clones obtained from libraries B, C, or D contained sequences that resembled mycoplasmas, despite the presence of co-migrating bands in the DGGE images. Furthermore, only library B (created from a digesta sample) was dominated (~92%) by Firmicutes, with 51 sequences showing 98% similarity to an uncultured *Clostridium* species (GenBank accession number EF092228). The second digesta sample yielded a clone

library (C) composed entirely of γ- and β-proteobacteria, with 60 of the sequences in this library showing 99% similarity to the β-proteobacterium *Ralstonia pickettii* (GenBank accession number EF195102). The tree created with sequences from clone library D consisted of ~9% Firmicutes and ~45% proteobacteria but was not dominated by a single phylotype. Not included in this tree were clones that most closely matched sequences from chloroplasts (~20% of the library), and clones that were determined to be contaminated by an isolate growing in the lab (~6%).

PIPEFISH (*SYNGNATHUS SCOVELLI*)

Intestinal tissue samples from different pipefish produced DGGE banding patterns that were very similar to each other, while DGGEs of digesta samples were more diverse (Fig. 5). In order to ensure that centrifugation harvested the complete microbial community, the supernatant was filtered through a Sterivex™ cartridge and extracted separately. This sample (denoted "1S" on figure 2) produced the same banding pattern as the sample from which it was taken. This sample was subsequently used to generate the first clone library (E) for the pipefish.

Twenty-four pipefish clones were sequenced; two of them did not return readable sequences. The majority of the sequences obtained was most similar to representative sequences from the class γ-proteobacteria: six (27%) of the sequences were most similar to various uncultured γ-proteobacteria, four (18%) were 97% similar to the γ-proteobacterium *Endozoicimonas elysicola*, and one (5%) sequence most closely resembled *Halomonas* sp. (94% similarity). Four clones contained sequences that were 95% similar to an uncultured α-proteobacterium, three (14%) were most closely related (99% similarity) to the β-proteobacterium *Delftia acidovorans*, and one (5%) was to the genus *Ruegeria*. There was one

clone each (5%) containing a sequence most similar to the following: *Acinetobacter* sp. (96% similarity), *Staphylococcus aureus* (96% similarity), and an unknown Spirochaete (94% similarity).

The sequences cloned from the three additional pipefish samples were similar to those obtained from the original sample, as seen in figure 7. All three clone libraries were dominated by members of the division proteobacteria, mostly α- and γ-proteobacteria. In library F, approximately 58% of the clones sequenced contained 16S rRNA genes most closely related to proteobacteria and only a single sequence (~1%) appeared to belong to the Firmicutes. This was the only pipefish library to contain any Firmicute sequences, and this sequence was only 94% similar to a species of *Clostridium*. The tree for library G contained approximately 52% proteobacteria sequences as well as nearly 20% that were most closely related to various members of the planctomycetes, which were not represented in the first pipefish clone library. Planctomycetes were also present in libraries D and F, but to a lesser extent (8% and 3%, respectively). In library H, sequences most similar to members of the proteobacteria were the most abundant (~56%). A sequence present in all three libraries was 99% similar to *Ralstonia pickettii* (GenBank accession number EF195102), the same β-proteobacterium that was most abundant in pinfish clone library C.

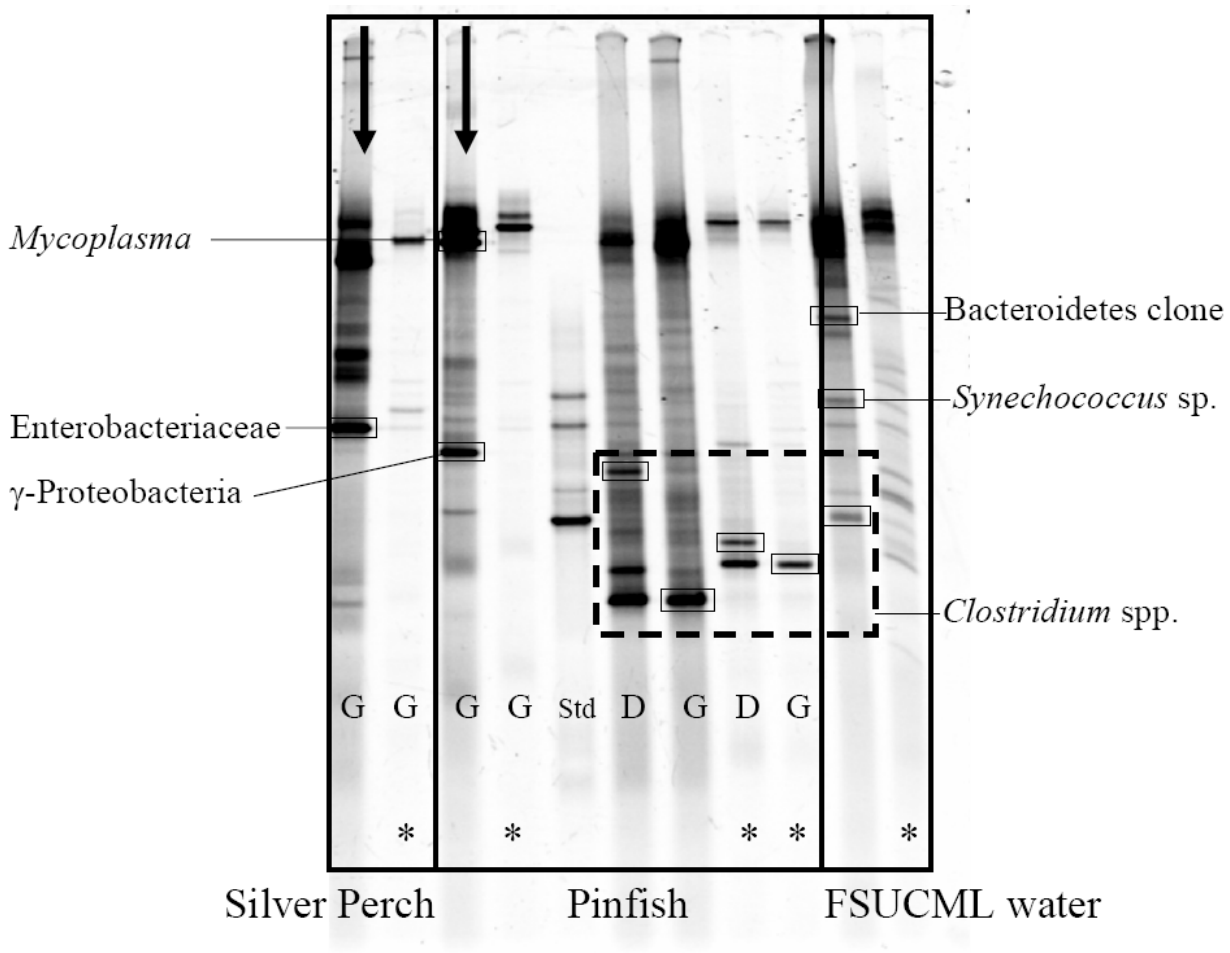


Figure 2. DGGE of silver perch and pinfish samples. Samples from gut tissue are marked with "G" and digesta samples are marked as "D." Arrows denote samples selected for the creation of a clone library. Lanes marked with an asterisk (*) contain DNA amplified with primers specific for the 16S rRNA genes from mycoplasmas, while the DNA in the other lanes was amplified with primers specific for Bacteria 16S rRNA genes.

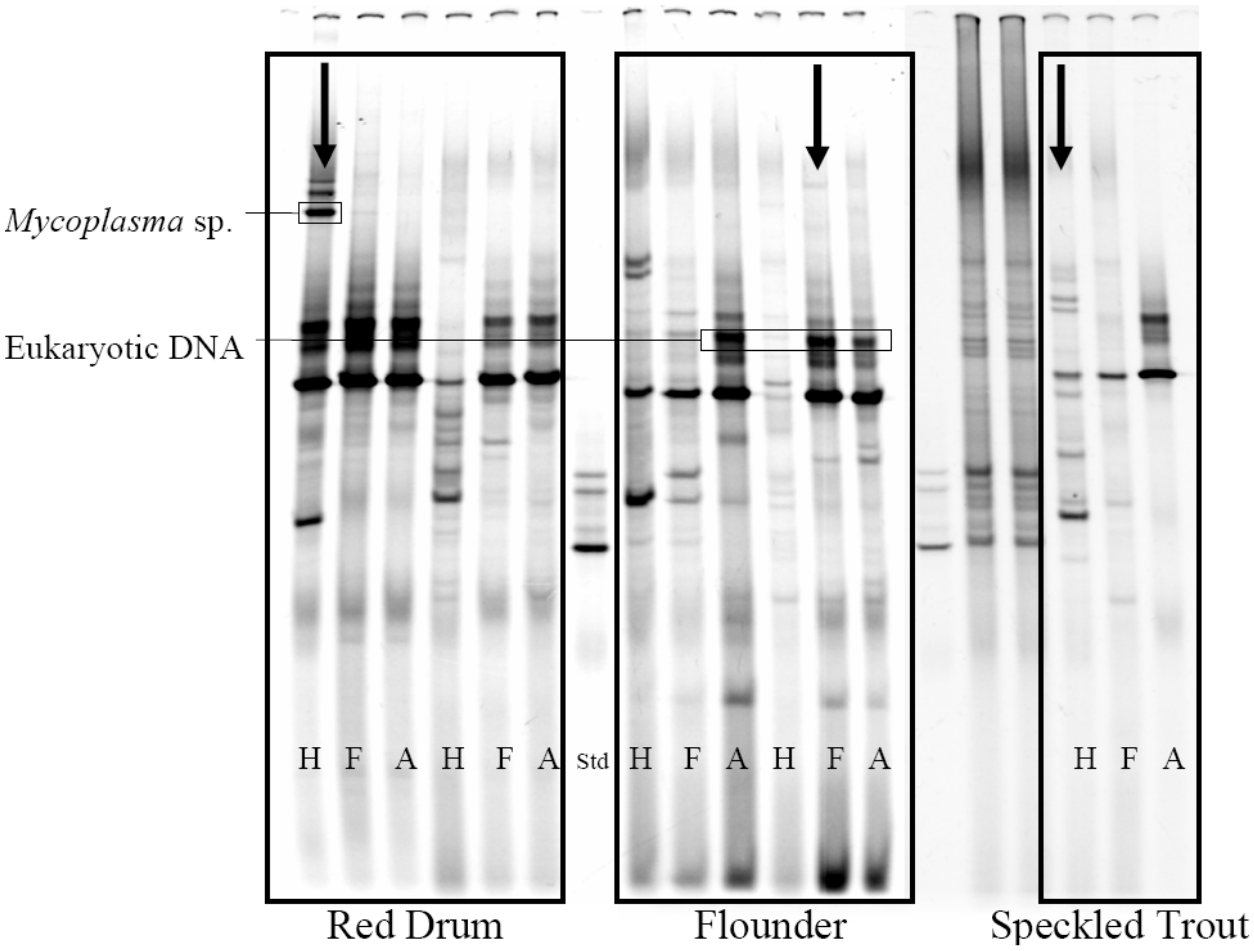


Figure 3. DGGE of red drum, flounder, and speckled trout samples that were amplified with primers specific for Bacteria 16S rRNA genes. Guts were divided into three sections: anterior (A), foregut (F), and hindgut (H). Arrows denote samples selected for the creation of clone libraries.

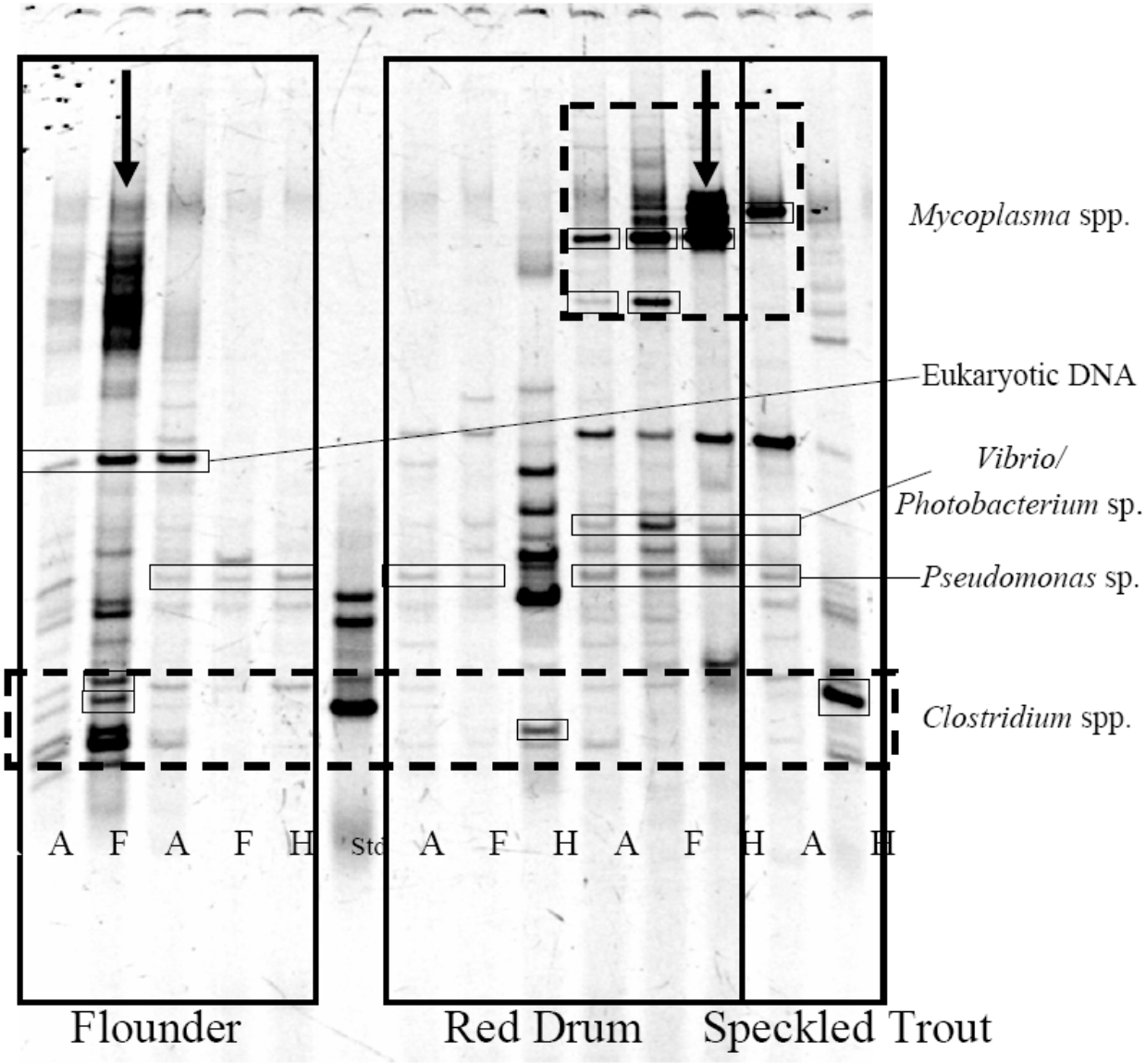


Figure 4. DGGE of red drum, flounder, and speckled trout samples that were amplified with primers specific for 16S rRNA genes from mycoplasmas. Guts were divided into three sections: anterior (A), foregut (F), and hindgut (H). Arrows denote samples selected for the creation of clone libraries.

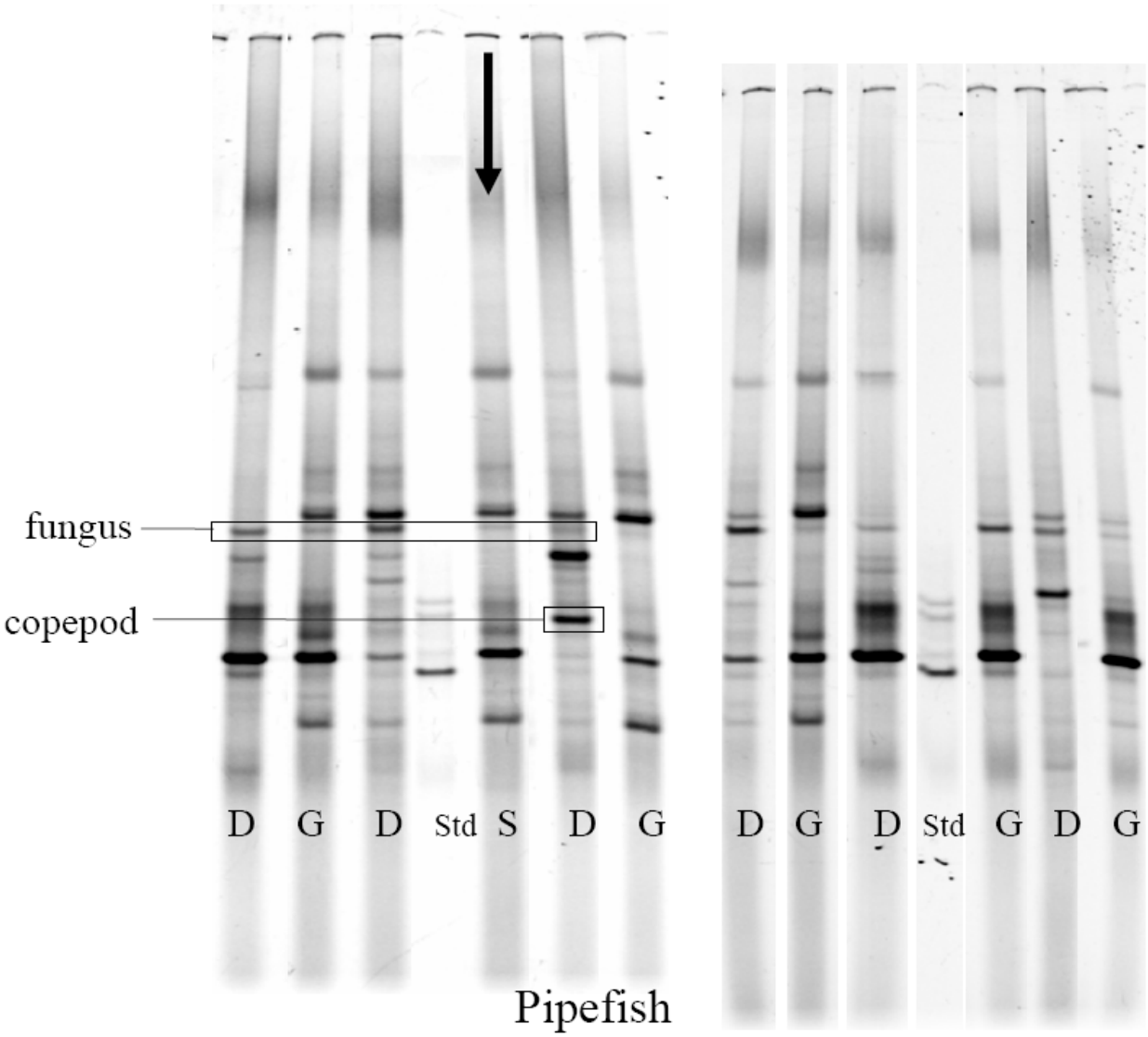


Figure 5. DGGE of pipefish samples that were amplified with primers specific for Bacteria 16S rRNA genes. Samples from gut tissue are marked with "G" and digesta samples are marked as "D." DNA extracted from the supernatant resulting from the centrifugation of fish gut tissue is marked "S." Arrow denotes sample selected for the creation of a clone library.

Figure 6. Neighbor-joining phylogenetic tree created with DNA sequences (~500bp) obtained from the clone libraries of pinfish samples. Representatives were chosen from clusters of sequences showing at least 98% similarity and relative abundance of each sequence in the library is indicated by the scale bar, color-coded for each library. Library A consisted of 23 sequences, library B contained 87 sequences, and there were 88 sequences each in libraries C and D. Bootstrap values are given where greater than 50% (100 iterations). The tree is unrooted with *Brevinema andersonii* (L31543) as the outgroup.

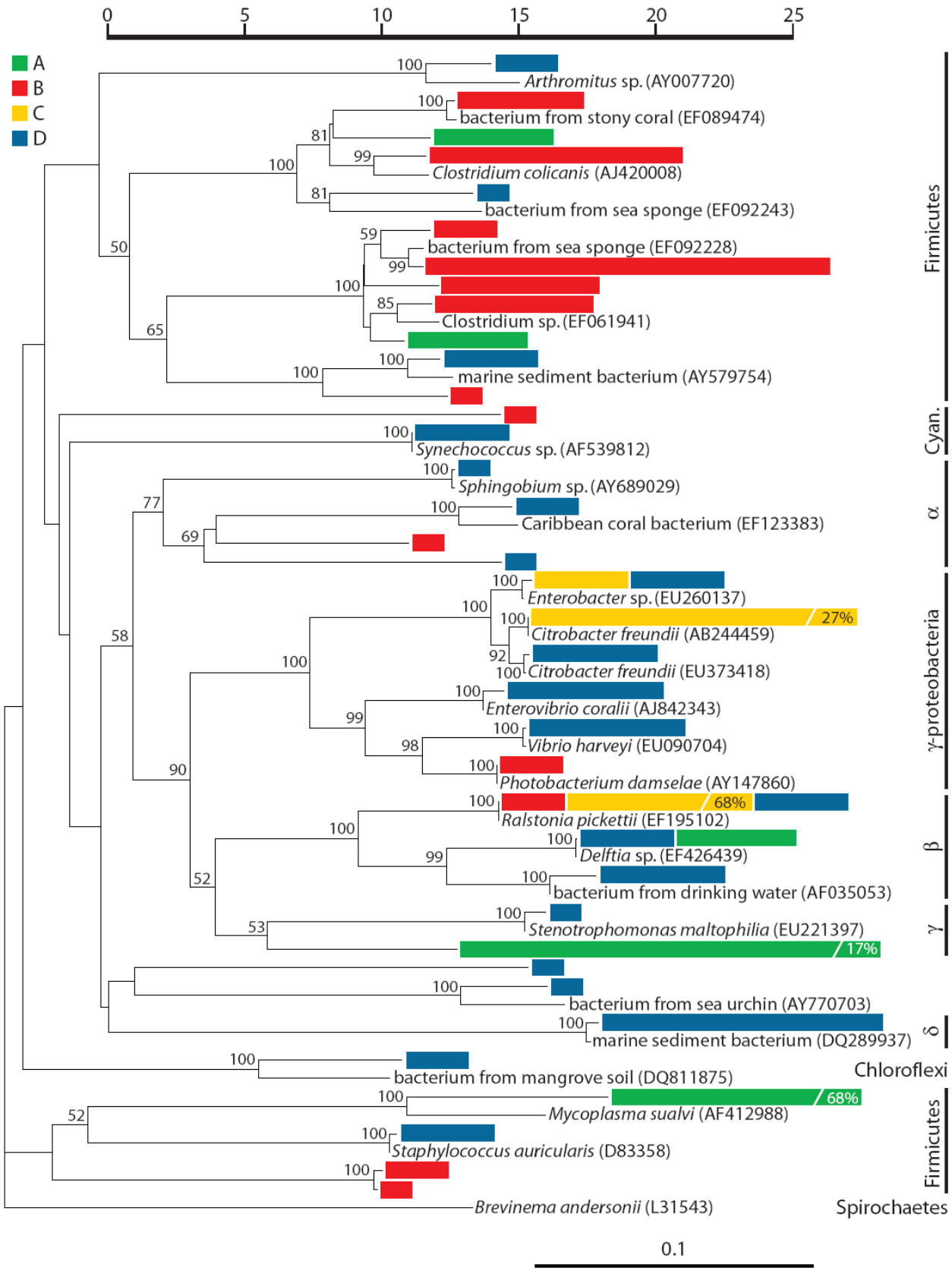
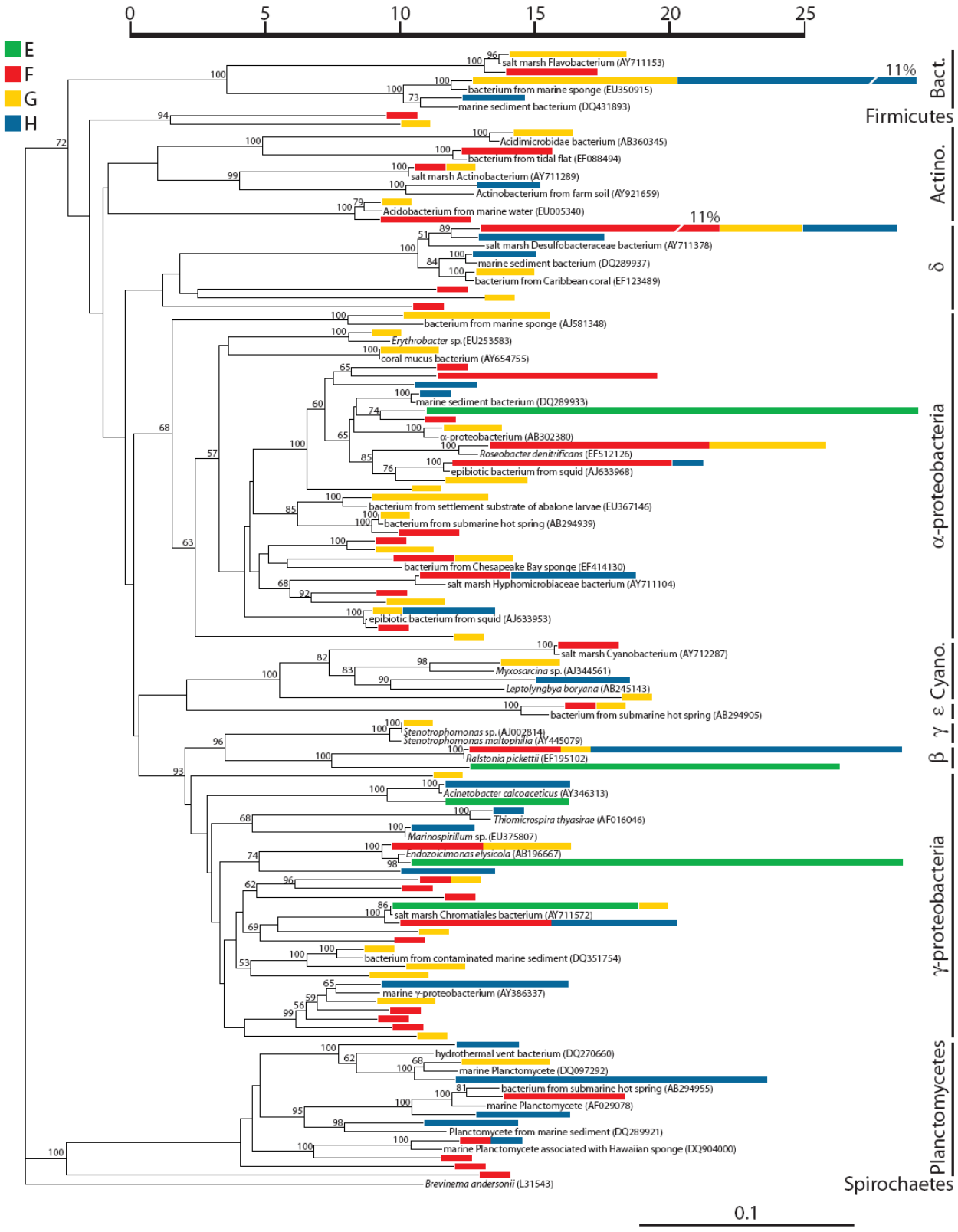

0
5
10
15
20
25
A
B
C
D
Arthromitus sp. (AY007720)
bacterium from stony coral (EF089474)
Clostridium colicanis (AJ420008)
bacterium from sea sponge (EF092243)
bacterium from sea sponge (EF092228)
Clostridium sp. (EF061941)
marine sediment bacterium (AY579754)
Synechococcus sp. (AF539812)
Sphingobium sp. (AY689029)
Caribbean coral bacterium (EF123383)
Enterobacter sp. (EU260137)
Citrobacter freundii (AB244459)
27%
Citrobacter freundii (EU373418)
Enterovibrio coralii (AJ842343)
Vibrio harveyi (EU090704)
Photobacterium damselae (AY147860)
Ralstonia pickettii (EF195102)
68%
Delftia sp. (EF426439)
bacterium from drinking water (AF035053)
Stenotrophomonas maltophilia (EU221397)
17%
bacterium from sea urchin (AY770703)
marine sediment bacterium (DQ289937)
bacterium from mangrove soil (DQ811875)
Mycoplasma sualvi (AF412988)
68%
Staphylococcus auricularis (D83358)
Brevinema andersonii (L31543)
Firmicutes
Cyan.
α
γ-proteobacteria
β
γ
δ
Chloroflexi
Firmicutes
Spirochaetes
0.1

Figure 7. Neighbor-joining phylogenetic tree created with DNA sequences (~500bp) obtained from the clone libraries of pipefish samples. Representatives were chosen from clusters of sequences showing at least 98% similarity and relative abundance of each sequence in the library is indicated by the scale bar, color-coded for each library. Library E consisted of 22 sequences, library F contained 90 sequences, there were 93 sequences in library G, and library H contained 87 sequences. Bootstrap values are given where greater than 50% (100 iterations). The tree is unrooted with *Brevinema andersonii* (L31543) as the outgroup.

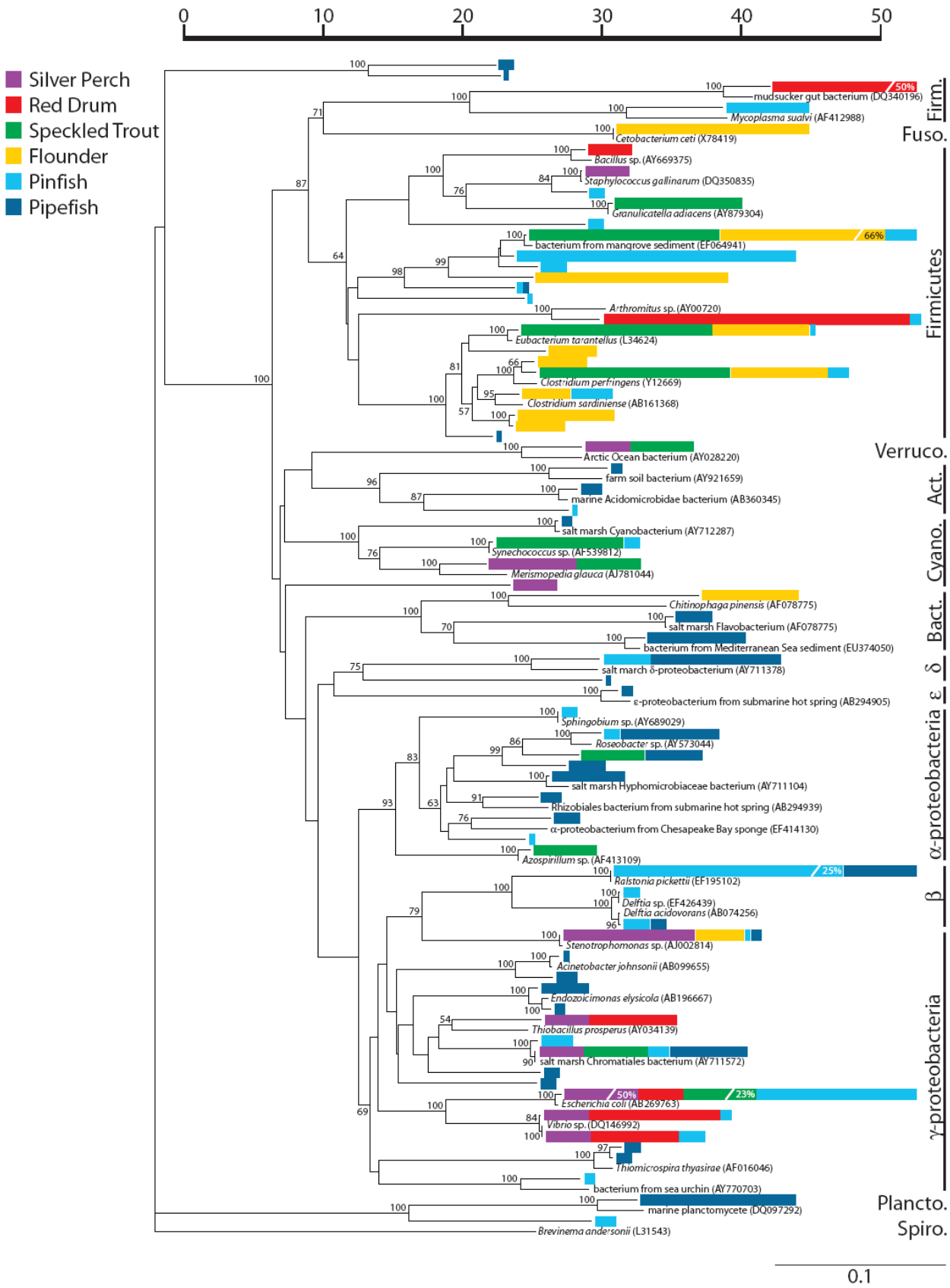

0
5
10
15
20
25
E
F
G
H
11%
11%
salt marsh Flavobacterium (AY711153)
bacterium from marine sponge (EU350915)
marine sediment bacterium (DQ431893)
Acidimicrobidae bacterium (AB360345)
bacterium from tidal flat (EF088494)
salt marsh Actinobacterium (AY711289)
Actinobacterium from farm soil (AY921659)
Acidobacterium from marine water (EU005340)
salt marsh Desulfobacteraceae bacterium (AY711378)
marine sediment bacterium (DQ289937)
bacterium from Caribbean coral (EF123489)
bacterium from marine sponge (AJ581348)
Erythrobacter sp. (EU253583)
coral mucus bacterium (AY654755)
marine sediment bacterium (DQ289933)
α-proteobacterium (AB302380)
Roseobacter denitrificans (EF512126)
epibiotic bacterium from squid (AJ633968)
bacterium from settlement substrate of abalone larvae (EU367146)
bacterium from submarine hot spring (AB294939)
bacterium from Chesapeake Bay sponge (EF414130)
salt marsh Hyphomicrobiaceae bacterium (AY711104)
epibiotic bacterium from squid (AJ633953)
salt marsh Cyanobacterium (AY712287)
Myxosarcina sp. (AJ344561)
Leptolyngbya boryana (AB245143)
bacterium from submarine hot spring (AB294905)
Stenotrophomonas sp. (AJ002814)
Stenotrophomonas maltophilia (AY445079)
Ralstonia pickettii (EF195102)
Acinetobacter calcoaceticus (AY346313)
Thiomicrospira thyasirae (AF016046)
Marinospirillum sp. (EU375807)
Endozoicimonas elysicola (AB196667)
salt marsh Chromatiales bacterium (AY711572)
bacterium from contaminated marine sediment (DQ351754)
marine γ-proteobacterium (AY386337)
hydrothermal vent bacterium (DQ270660)
marine Planctomycete (DQ097292)
bacterium from submarine hot spring (AB294955)
marine Planctomycete (AF029078)
Planctomycete from marine sediment (DQ289921)
marine Planctomycete associated with Hawaiian sponge (DQ904000)
Brevinema andersonii (L31543)
Bact.
Firmicutes
Actino.
δ
α-proteobacteria
Cyano.
ε
γ
β
γ-proteobacteria
Planctomycetes
Spirochaetes
0.1

CHAPTER 4

DISCUSSION

There is a great diversity of intestinal microbiota between different fish species. While there does not appear to be a "typical" microbial community common to all fish, the gut microflora of the fish species studied thus far seem to be dominated either by members of the proteobacteria or the Firmicutes. Figure 8 shows the phylogenetic relationship between bacterial species and their relative abundance in the clone libraries from each fish species, indicated by the colored scale bar. LIBSHUFF analysis of these clone libraries indicated that each was significantly different. In this study the pipefish and silver perch were both dominated by proteobacteria, while the red drum and flounder were dominated by Firmicutes. Although results from the initial pinfish clone library (A) suggested that Firmicutes, and mycoplasmas in particular, made up a significant portion of the intestinal microflora, after the addition of

Figure 8. Neighbor-joining phylogenetic tree created with DNA sequences (~500bp) obtained from the clone library of each fish species. Representatives were chosen from clusters of sequences showing at least 98% similarity and relative abundance of each sequence in the library is indicated by the scale bar, color-coded for each fish species. Bootstrap values are given where greater than 50% (100 iterations). The tree is unrooted with *Brevinema andersonii* (L31543) as the outgroup.


0
10
20
30
40
50
Silver Perch
Red Drum
Speckled Trout
Flounder
Pinfish
Pipefish
mudsucker gut bacterium (DQ340196)
Mycoplasma sualvi (AF412988)
Cetobacterium ceti (X78419)
Bacillus sp. (AY669375)
Staphylococcus gallinarum (DQ350835)
Granulicatella adiacens (AY879304)
bacterium from mangrove sediment (EF064941)
Arthromitus sp. (AY00720)
Eubacterium tarantellus (L34624)
Clostridium perfringens (Y12669)
Clostridium sardiniense (AB161368)
Arctic Ocean bacterium (AY028220)
farm soil bacterium (AY921659)
marine Acidomicrobidae bacterium (AB360345)
salt marsh Cyanobacterium (AY712287)
Synechococcus sp. (AF539812)
Merismopedia glauca (AJ781044)
Chitinophaga pinensis (AF078775)
salt marsh Flavobacterium (AF078775)
bacterium from Mediterranean Sea sediment (EU374050)
salt march δ-proteobacterium (AY711378)
ε-proteobacterium from submarine hot spring (AB294905)
Sphingobium sp. (AY689029)
Roseobacter sp. (AY573044)
salt marsh Hyphomicrobiaceae bacterium (AY711104)
Rhizobiales bacterium from submarine hot spring (AB294939)
α-proteobacterium from Chesapeake Bay sponge (EF414130)
Azospirillum sp. (AF413109)
Ralstonia pickettii (EF195102)
Delftia sp. (EF426439)
Delftia acidovorans (AB074256)
Stenotrophomonas sp. (AJ002814)
Acinetobacter johnsonii (AB099655)
Endozoicimonas elysicola (AB196667)
Thiobacillus prosperus (AY034139)
salt marsh Chromatiales bacterium (AY711572)
Escherichia coli (AB269763)
Vibrio sp. (DQ146992)
Thiomicrospira thyasirae (AF016046)
bacterium from sea urchin (AY770703)
marine planctomycete (DQ097292)
Brevinema andersonii (L31543)
50%
66%
25%
50%
23%
Firm.
Fuso.
Firmicutes
Verruco.
Act.
Cyano.
Bact.
δ
ε
α-proteobacteria
β
γ-proteobacteria
Plancto.
Spiro.
0.1

sequences from the replicate clone libraries (B, C, and D) nearly 55% of the sequences obtained from pinfish intestinal samples were determined to be proteobacteria. The speckled trout did not appear to be dominated by either Firmicutes or proteobacteria. This could be due to the fact that only one speckled trout specimen was available at the time of analysis and investigation of a larger number of individuals would provide a more robust characterization of the typical microbial community found in speckled trout intestines.

After creating replicate clone libraries with three additional pinfish samples, the presumed dominance of Firmicutes as important gut microflora in this species became less clear. Library B was clearly dominated by Firmicutes, particularly sequences closely related to bacteria belonging to the genus *Clostridium*. Library D contained a total of 9 Firmicute sequences, and again there were four sequences that were most closely related to members of Clostridiales. Library C contained no Firmicutes; all sequences were most similar to members of γ- and β-proteobacteria. LIBSHUFF analysis of libraries B, C, and D confirmed that they were significantly different from each other.

The marked difference between the intestinal microflora of the four different individual pinfish was unexpected. However, research has shown that juvenile pinfish prey on small invertebrates (e.g. copepods, amphipods, and polychaetes) and consume increasing amounts of plant matter as they mature (Luczkovich and Stellwag 1993; Gallagher, Luczkovich et al. 2001). This shift in diet is accompanied by a change in the gut microflora; pinfish larger than 40 mm standard length contained a larger percentage (13-50%) of bacteria capable of hydrolyzing carboxomethylcellulose than individuals smaller than 40 mm (Luczkovich and Stellwag 1993). The structure of the digestive tract also changes as pinfish mature. The intestinal tract elongates and gastric glands not found in juveniles under 40 mm standard length become numerous in

adults (Gallagher, Luczkovich et al. 2001). Because of the shift in diet, it is possible that different individuals within the same species could harbor distinctly different microbial communities within their digestive tracts. However, pinfish used in this study were all significantly larger than 40 mm and were presumably herbivorous adults.

Common bacterial genera previously found in the intestines of freshwater and marine fish include *Enterobacter*, *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, and *Vibrio* (Cahill 1990; Ringø, Strøm et al. 1995). With the exception of *Flavobacterium*, all of these organisms belong to the proteobacteria. It is interesting to note that these identifications were based on cultivation-dependent methods, in contrast to other recent studies. One of the early studies of fish intestinal microflora that used molecular methods demonstrated an abundance (up to 96% of total microbial community) of a *Mycoplasma* phylotype in salmon (Holben, Williams et al. 2002) and a recent characterization of mudsucker (*Gillichthys mirabilis*) gut microflora showed a dominance of mycoplasmas, one of which was very similar to the phylotype found in the salmon (Bano, Smith et al. 2007). Although notoriously difficult to grow in pure culture, mycoplasmas were found to be associated with fish prior to the use of molecular identification methods. *Mycoplasma mobile* was isolated from the gills of a tench (*Tinca tinca*) with red disease (Kirchhoff and Rosengarten 1984; Kirchhoff, Beyene et al. 1987) and *Acholeplasma laidlawii* was detected in emaciated centrarchids in Lake Harris, FL, although the researchers concluded it was not pathogenic (Francis-Floyd, Reed et al. 1998). The focus of these earlier culture-based studies was on diseased fish and not gut microflora.

The molecular methods in this study also suggested an abundance of mycoplasmas in some fish species, specifically the pinfish and the red drum. However, the mycoplasma-like organisms detected in the clone libraries for these two species were only approximately 92-93%

similar to published mycoplasma 16S rRNA gene sequences. Furthermore, upon the creation of replicate pinfish clone libraries no mycoplasma species were detected. Since mycoplasmas have such a reduced genome, they are typically parasites or commensals in a host organism and often attach to the tissue of the host (Balish and Krause 2006; Kostanjšek, Štrus et al. 2007), which could explain why they were not detected in the digesta samples. The method of collecting digesta (squeezing it out from an intact intestine) from the pinfish could have left behind host cells to which the presumed mycoplasmas were attached. The third pinfish sample, however, did include fish gut tissue but still did not yield any mycoplasma sequences. While this is obviously an area where more research needs to be done before drawing a final conclusion, it seems likely that if mycoplasmas are a dominant gut resident then they would be present in all clone libraries created from four different individual fish, which was not the case in this study.

While molecular identification methods have helped characterize Firmicute gut microflora that traditional culture-based methods may have missed, they have also shown that some fish species simply do not have a large Firmicute component to their intestinal microbial community (Asfie, Yoshijima et al. 2003; Huber, Spanggaard et al. 2004; Rawls, Samuel et al. 2004; Skrodenyte-Arbaciauskiene, Sruoga et al. 2006; Smith, Danilowicz et al. 2007). In the current study, the initial pipefish and silver perch samples were both shown to have intestinal microbiota dominated by proteobacteria. The replicate pipefish libraries confirmed this pattern, although LIBSHUFF analysis indicated that these libraries were significantly different from each other. The most abundant sequences present in all libraries were most closely related to species belonging to the α- and γ-proteobacteria.

Gut morphology of different fish species is highly variable (Suyehiro 1942) and seems to be a function of diet. Herbivorous fishes require a longer alimentary canal than carnivores

(Cahill 1990; Helfman, Colette et al. 1997; Gallagher, Luczkovich et al. 2001; Kapoor and Khanna 2004; Moran, Turner et al. 2005). All of the fish in this study are typically carnivorous, with the exception of the omnivorous pinfish that incorporate an increasing amount of plant and algal matter into their diet as they mature. Other researchers have shown Firmicutes, specifically members of the *Mycoplasma* genus, to dominate the intestines of carnivorous/omnivorous fish such as wild- and pen-raised Scottish salmon (Holben, Williams et al. 2002) and mudsuckers, *Gillichthys miribalis* (Bano, Smith et al. 2007). Carnivorous/omnivorous fish whose intestines appear to be dominated by proteobacteria include: rainbow trout, *Oncorhynchus mykiss* (Spanggaard, Huber et al. 2000; Huber, Spanggaard et al. 2004), goldfish, *Carassius auratus* (Asfie, Yoshijima et al. 2003), zebrafish, *Danio rerio* (Rawls, Samuel et al. 2004; Rawls, Mahowald et al. 2006), river trout, *Salmo trutta* (Skrodenyte-Arbaciauskiene, Sruoga et al. 2006), and whiting, *Merlangius merlangus* (Smith, Danilowicz et al. 2007). Previous research with strictly herbivorous fish using molecular techniques showed a dominance of Firmicutes (particularly members of the genus *Clostridium*) in the silver drummer, *Kyphosus sydneyanus* (Moran, Turner et al. 2005; Clements, Pasch et al. 2007). In contrast, the gut of the herbivorous zebraperch, *Hermosilla azurea*, was dominated by proteobacteria belonging to the genus *Enterovibrio* (Fidopiastis, Bezdek et al. 2006). Thus, the current body of research does not suggest a clear correlation between diet and the composition of the gut microflora.

While a carnivorous versus herbivorous diet does not seem to determine the composition of the microbial community in fish intestines, there is evidence that the gut environment plays an important role in shaping the community structure. In the DGGE gel comparing pinfish gut microflora with the bacteria in the surrounding water column (Fig. 2), the banding pattern for the water sample is distinctly different from the banding pattern observed in the pinfish intestinal

samples. Previous studies have shown that the composition of the gut microflora differs from the surrounding water, with the gut microflora often consisting of a much simpler community (Cahill 1990; Ringø, Strøm et al. 1995; Smith, Danilowicz et al. 2007). It is clear that the composition of the microbial community within the intestine of a fish is not simply a function of the bacteria in the surrounding water. Experiments with zebrafish have shown that when mouse intestinal microflora were transplanted into germ-free fish, the composition of the bacterial community shifted to reflect the relative abundance normally found in the zebrafish (Rawls, Mahowald et al. 2006). This experiment demonstrates that the gut of a fish is a selective environment that is capable of harboring a unique microbial community.

CHAPTER 5

CONCLUSION

In this survey of the intestinal microbiota of six different southeastern fish species, it is clear that there is not a typical bacterial community that resides within the digestive tracts of different fishes. It appears that some fish intestines are dominated by members of the division proteobacteria as characterized by traditional culture-based identification methods as well as modern molecular methods. However, newer methods have also detected an abundance of Firmicutes over proteobacteria in some fish species. These molecular methods, such as PCR/DGGE analysis and cloning, have enabled researchers to identify microorganisms previously unknown in intestinal microflora. For example, it is only recently that mycoplasmas have been identified as important residents in the guts of fish such as salmon (Holben, Williams et al. 2002) and mudsuckers (Bano, Smith et al. 2007).

This survey raises interesting questions about the fundamental composition of fish gut microbial communities, particularly concerning the dominance of proteobacteria versus Firmicutes. While both carnivorous and herbivorous fish can be dominated by one group or the other, further feeding experiments may be able to determine the effect of specific food items on intestinal microflora. The beneficial effects of gut commensals, especially in the breakdown of plant matter undigestable by the fish, have been documented in a number of studies (Stellwag, Smith et al. 1995; Mountfort, Campbell et al. 2002; Moran, Turner et al. 2005). The possibility of Firmicutes playing an important role in the nutrition of the fish is an area that should be

studied further. A recent study of mice intestinal microflora suggests that the relative abundance of Firmicutes over Bacteroidetes results in more efficient energy recovery from food, thus obese mice had a higher percentage of Firmicutes in their guts than their lean counterparts (Turnbaugh, Ley et al. 2006). While members of Bacteroidetes were not dominant in the intestinal microflora of the fish in this study (as they are in mice), Firmicutes were abundant in the pinfish, red drum, and flounder. Therefore, the varying proportion of Firmicutes relative to other intestinal endosymbionts warrants a closer look at the effect of diet on gut microflora.

While this survey has yielded some interesting results, there is still much to be learned about the intestinal microbiota of fish. The role that many of these organisms play in fish nutrition is unknown, yet the intestinal community differs from that of the surrounding water, suggesting an ecological niche for these organisms. By applying methods such as those described in this study to a greater variety of fish species, it may be possible to expand our knowledge of gut microflora and better understand the relationship between the microorganisms and the fish they inhabit.

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